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STUDIES ON THE LIFE HISTORY OF *EURYTREMA PROCYONIS* DENTON, 1942 [1]

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INTRODUCTION

Knowledge of the life cycles of trematodes of the subfamily Dicrocoeliinae Looss, 1899, exists for a single species, *Dicrocoelium dendriticum* (Rudolphi, 1819). The cercaria, *Cercaria vitrina* von Linstow, 1887, of this species has been known for years, but only recently has its complete development in the molluscan hosts been studied and its relationship to the adult proven. Vogel (1929) described previously unreported anatomical details of *C. vitrina* and concluded, on the basis of morphological similarity, that it was the cercaria of *D. dendriticum.* Cameron (1931) obtained cercariae which he identified as *C. vitrina* from snails, *Helicella itala* (= *H. ericetorum* Geyer, 1927), to which he had fed *D. dendriticum* eggs. Mattes (1933, 1936) observed the complete development of *C. vitrina* in snails, *H. ericetorum, H. candidula,* and *Zebrina detrita,* which he infected by feeding eggs. Finally, with the observations of Neuhaus (1936, 1938) of the encystment of the cercariae and the method of infection of the definitive hosts, the life cycle of *D. dendriticum* was completed.

Hoping to acquire knowledge of the life histories of additional species of the subfamily Dicrocoeliinae and possibly to determine the taxonomic value of larval characters for this group, a number of attempts were made between 1938 and 1941 to experimentally determine the life cycles of the common dicrocoeliids occurring in hosts in the vicinity of Houston, Texas. The present account, the first of a series, records results of the experiments on the life cycle of *Eurytrema procyonis* Denton, 1942.

MATERIALS AND METHODS

From knowledge of the life cycle of *Dicrocoelium dendriticum,* it seemed likely that the molluscan host of *Eurytrema procyonis* would be a land snail. So a survey was made to determine the terrestrial snails occurring in Southeastern Texas. Living specimens of seven of the more common species were brought to the laboratory and breeding cultures were set up according to the methods described by Krull (1937). Excellent results were obtained in breeding [illegible] seven species. However, *Bulimulus alternatus mariae* grew too slowly in the laboratory for our purposes, so specimens for use in experiments were collected from a heavily populated area

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where the snails were free of trematode infections as determined by examination of over 1,000 individuals.

Eggs used in the feeding experiments were obtained from the uteri of mature worms. After incubating the worms in tap water at 38–39° C for 48 hours, the embryonated eggs were teased out in a drop of water on a slide and examined to make certain that the miracidia were alive. A little cornmeal was then added and the eggs were fed to groups of 10 to 25 snails. To prevent the infection from being too heavy and killing the snails, an average of only 10 eggs per snail was fed. After 12 to 18 hours, droppings from the snails were examined for evidence of hatching. The snails were then placed in permanent cultures and kept for three months before being determined negative for infection. During the course of all experiments, the snails were given abundant food and the temperature of the laboratory remained at 18 to 25° C.

Sporocysts and cercariae were studied alive unstained, or stained *intra vitam* with neutral red and Nile blue sulphate. Measurements and sketches given in connection with this paper, unless otherwise stated, were made from fresh material killed with gentle heat. For permanent mounts, sporocysts and cercariae were fixed in hot Gilson's or Bouin's fixatives and stained with carmalum. Detail studies were made with the aid of either a high dry or an oil immersion lens.

BIOLOGY AND LIFE HISTORY

The adult *Eurytrema procyonis* is a common parasite in the interlobular ducts of the pancreas of raccoons, *Procyon lotor,* in Southeastern Texas. Six of ten animals examined from that locality harbored from 12 to more than 1,000 specimens of this trematode. The heavily infected raccoons showed no discernible effects from their infections.

Attempts to induce adult worms to discharge their mature eggs in physiological saline or tap water were unsuccessful. Also, many attempts to hatch eggs outside of the digestive tract of snails failed. Such attempts included incubation in tap water and 0.5 per cent saline at room temperature for various lengths of time, incubation in various dilutions of pepsin, and incubations in fluid from the stomach and intestine of snails.

Eggs of this worm were fed to *Polygyra texasiana* (Moricand), *Mesodon thyroidus* (Say), *Practicollela berlandieriana* (Moricand), *Angiuspira alternata* (Say), *Bulimulus alternatus mariae* (Albers), and *Deroceras agrestis* (Linnaeus). The eggs hatched in all six species of snails but development proceeded only in *Mesodon thyroidus.* Attempts to study living miracidia from the intestines of these snails were unsuccessful. The partly digested cornmeal produced a milky medium in which it was very difficult to observe the hatched miracidia. As well as could be determined all mature eggs had hatched after two hours, the hatching occurring in the upper small intestine of the host. The miracidia penetrated the intestinal wall in this region and passed to the middle digestive gland to continue their development.

Of 10 adult *M. thyroidus* to which eggs of *E. procyonis* were fed, seven became heavily infected, two failed to become infected, and one examined after 50 days may or may not have been infected.

It is probable that the developing mother sporocysts of *E. procyonis* can be detected with the dissecting microscope by the 50th day of infection. However, since *M. thyroidus* was not known to be a suitable host at that time, only one snail was sacrificed with negative results. A second specimen, examined on the 70th day of infection, contained several mother sporocysts which were just maturing. These sporocysts were located in the haemocoelic space, deep between the lobes of the middle digestive gland and contained daughter sporocysts in all stages of development from undifferentiated germ balls to recognizable sporocysts. Approximately 100 daughter sporocysts which had already ruptured from the parent forms were present in the digestive gland and along the adjacent intestine. In a snail autopsied on the 95th day of infection, the mother sporocysts were still intact and contained daughter sporocysts in all stages of development. In addition, over 2,000 daughter sporocysts in various stages of development were distributed throughout the digestive gland and other tissues of the snail.

Although no mother sporocysts were observed in snails examined on later dates, the evidence indicates that mother sporocysts produce daughter sporocysts continuously over a period of a month and probably longer, rather than a single generation of daughters which mature and rupture simultaneously from the parent.

The daughter sporocysts, after escaping from the parent, gradually migrate toward the respiratory portion of the mantle and the exposed parts of the snail's body. In a snail autopsied on the 95th day, the majority of these sporocysts were still concentrated in the digestive gland, although some of the largest and most developed ones had reached the wall of the mantle cavity where they were found in the branches of the pulmonary vein. Other daughter sporocysts were found in clusters in the collar and in the softer dorsal tissues of the body. In another snail examined on the 115th day, the branches of the pulmonary vein were greatly distended with daughter sporocysts. The collar and adjoining tissues were packed with them, and the foot, even the toughest part of it, contained many.

Some of the daughter sporocysts matured and were expelled on the 141st day of infection. From then until the experiment was terminated on the 177th day, the snails continued to expel masses of mature sporocysts daily. Observation of the snails revealed that the mature sporocysts rupture into the mantle cavity where they collect in groups of from two to three to as many as 125. The surfaces of the sporocysts are sticky, causing them to clump together. These clumps then become covered with mucus before being expelled from the respiratory pore of the host. As the snails crawl about, the expelled sporocysts are deposited on plants and other objects. In the humid atmosphere of the culture, sporocysts were expelled any time of day or night. Whether or not specific environmental factors affect the expulsion of sporocysts by snails was not determined.

When sporocysts are first expelled by snails, they are glistening white in appearance. After a short time the sporocyst wall dehydrates and shrivels, turning a light yellowish-brown color. This dead wall does not disintegrate but persists intact as a protective covering around the endocyst which encloses the cercariae. Cercariae in such sporocysts, deposited in a humid atmosphere or on the leaves of transpiring plants, remain alive for two or three days before disintegrating. In sporocysts placed in the refrigerator in 0.5 per cent saline, some cercariae were alive after five days.

At no time were cercariae found outside of the sporocyst in which they developed. Within the fluid which fills the body cavity of expelled sporocysts, the cercariae slowly extend and contract. Mechanical disturbance or immersion of the sporocyst in 0.5 per cent saline causes the cercariae to become more active. When the sporocyst wall and endocyst are ruptured, the freed cercariae crawl over the bottom of the container in geometrid fashion. The surface of the tail is sticky and appears to serve as an adhesive organ. After a few minutes of activity the cercariae become exhausted and lie motionless in the dish. They die in 6–8 hours.

At this point the question arose as to whether or not a second intermediate host is involved in the life cycle of this worm. Examination of two uninfected adult *M. thyroidus* which had been exposed for 25 days with the individuals expelling sporocysts daily, failed to reveal either free or encysted cercariae. Three half-grown *M. thyroidus* exposed for 14 days with snails expelling sporocysts also failed to show any cercariae. It was evident that *M. thyroidus* does not serve as a second intermediate host for this worm and it is unlikely that any other snail does. Whether or not some other animal serves in this capacity is still undetermined.

The fact that cercariae leave the molluscan host imprisoned in and protected by a tough endocyst suggested that infection of the definitive host may occur directly from eating snails containing mature sporocysts. Unfortunately, circumstances beyond our control abruptly terminated the experiments before this hypothesis could be tested. However, it was definitely determined (unpublished data) that in a *Eurytrema* from birds, direct infection of the definitive host does not occur.

DESCRIPTION OF DEVELOPMENTAL STAGES

The egg and miracidium.—The embryonated egg of *E. procyonis* (Fig. 1) is oval in outline, lemon-yellow in color, operculated, and measures 45–53 μ long by 29–36 μ wide. The lightly bile-stained shell is fairly thick but transparent. Usually many refractile droplets, probably excretory granules or disintegrating yolk, are present in the space between the shell and miracidium, particularly at the anterior end. The fully developed larva, orientated toward the operculum, exhibits practically no movement. Its body is pear-shaped and covered with cilia, most numerous and longest at the anterior end. Protruding slightly from the anterior end and extending posteriorly about one-third of the body length is a slender stylet. Within the anterior half of the body, the vague outline of the primitive gut and penetration glands can be detected. Two large, oval, oppositely placed vesicles, containing highly refractile granules, occupy most of the posterior half of the body. Between the gut and the refractile bodies several large cells, believed to be germ cells, are discernible. No epidermal plates or flame cells could be detected in either unhatched miracidia or miracidia forced from eggs by pressure.

The mother sporocyst.—The mother sporocyst as observed on the 70th day of infection is a pearly white, extensively lobed mass. Apparently, its shape is determined somewhat by the location in which it develops. It is of a very delicate texture and firmly attached to the tough connective tissue of the host, making separation very difficult. Externally it is covered by a thin delicate membrane composed of a single layer of flattened epithelial cells. Whether or not a thin cuticle is present was not determined. The mass of the body or matrix is composed of irregularly shaped cells with small round nuclei. The numerous developing daugh-

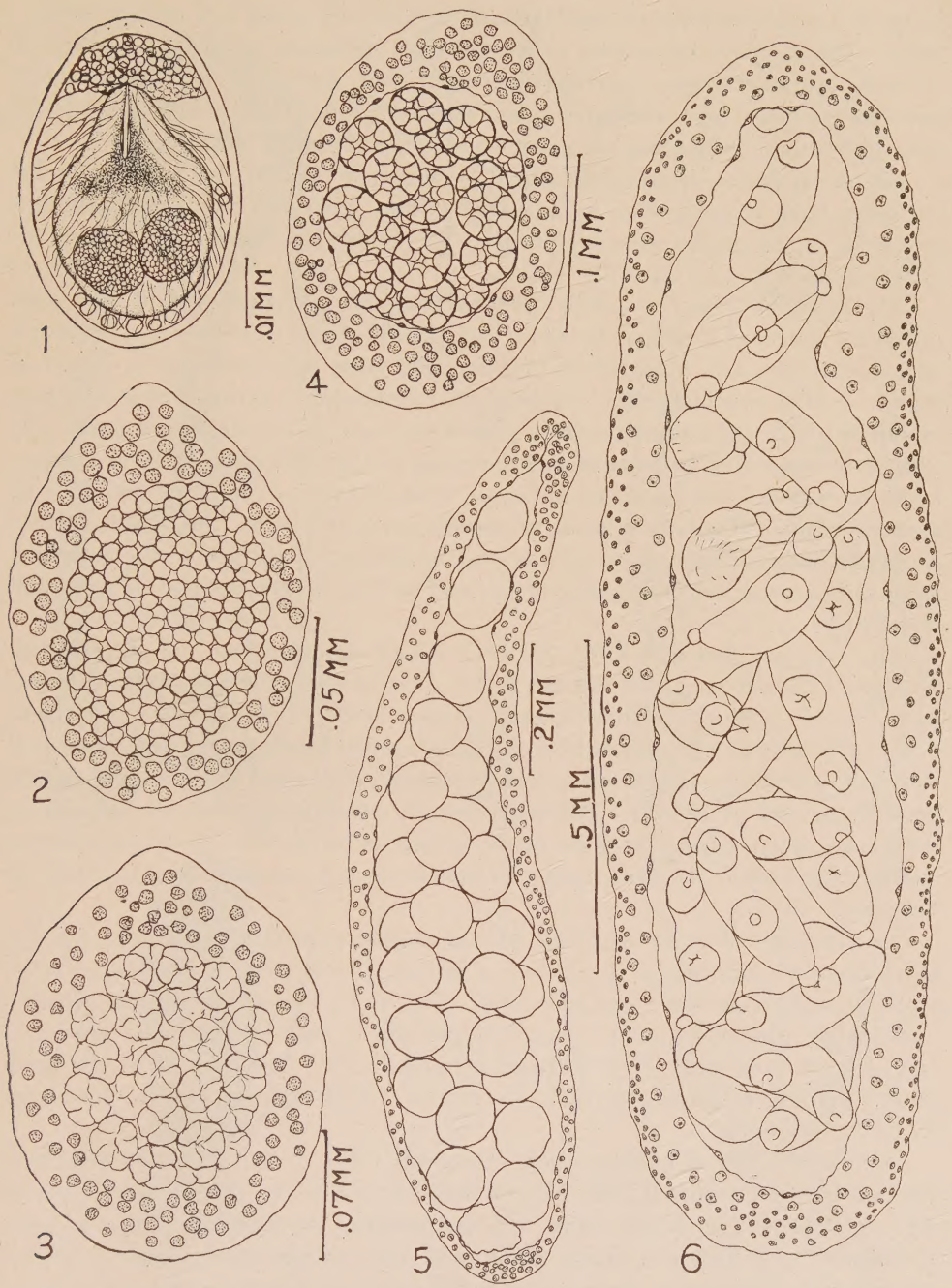

FIG. 1. Egg with mature miracidium, showing stylet and large refractive bodies.

FIG. 2. Immature daughter sporocyst recently ruptured from mother sporocyst, showing outer somatic portion and central mass of germ cells.

FIG. 3. Daughter sporocyst showing segmentation of the germ cell mass into cercarial "germ balls."

FIG. 4. Daughter sporocyst after formation of cercarial embryos and appearance of epithelial lining of body cavity.

FIG. 5. Daughter sporocyst on the 95th day of infection.

FIG. 6. Mature daughter sporocyst containing fully formed cercariae.

ter sporocysts are scattered through the matrix in cavities which lack definite cellular walls. The daughter sporocysts, which are most numerous near the periphery of the lobes, are in all stages of development from small germ balls to forms ready to leave the parent. Since daughter sporocysts were found free in the haemocoelic space at this time, the mother sporocyst was assumed to be mature.

Mother sporocysts observed in a snail examined on the 95th day of infection did not differ from those studied on an earlier date. They also contained daughter sporocysts in all stages of development. No mother sporocysts were found in snails autopsied at later dates.

The daughter sporocyst.—The youngest daughter sporocyst embryo contained within the matrix of a mother was in the "germ ball" stage. This embryo measured 18 μ in diameter and consisted of four rounded germ cells enclosed by about 16 slightly smaller cuboidal cells. Various other embryos, in more developed stages, contained within the mothers, show a gradual increase in the number of germ cells with a corresponding increase in and flattening of the outer cells to form a membrane or wall one cell layer in thickness. An embryo which measured 35 μ in diameter contained a central mass of approximately 16 germ cells. Still further developed daughter sporocysts show a gradual increase in the size of the germ cell mass with a concurrent thickening of the wall so that it becomes two or three cell layers thick.

The daughter sporocyst (Fig. 2), when it first ruptures from the parent form, averages 0.273 mm long by 0.150 mm wide. It is an oval, colorless, delicate, transparent form capable of slight movement. It is covered by a thin cuticle 1 μ in thickness. The body is differentiated into an outer somatic portion consisting of several layers of irregularly shaped cells, and a central germ mass consisting of tightly packed, rounded germ cells. Although there is no definite membrane separating the somatic portion from the germ mass, the line of demarcation is very distinct in stained specimens. No flame cells or elements of the excretory system could be detected.

In somewhat older forms (Fig. 3) the somatic portion is unchanged but the germ mass is beginning to divide into a number of cercarial germ balls of equal size. In still further developed sporocysts (Fig. 4) the soma is completely separate and the body cavity resulting from this separation is lined by a definite membrane consisting of a single layer of flattened epithelial cells. The division of the germ mass into round cercarial germ balls is complete. Each cercarial embryo is covered by a thin membrane.

By the 95th day of infection these sporocysts (Fig. 5) had increased greatly in size, the largest measuring 1.42 mm long and 0.40 mm wide. The cuticle measured 1.5 μ in thickness, while the entire wall, composed of three or four layers of cells, averaged 25 μ in thickness except at the ends where it was much thicker. Within the relatively transparent walls of the living sporocyst many elements of a paired excretory system are now visible. The two excretory pores are located in opposite walls near the middle of the sporocyst. Just interior to each excretory pore is a small sinus from which arise four main collecting tubules. One main tubule runs anteriorly to supply the anterior end, another runs posteriorly to supply the opposite end, while the other two run laterally in opposite directions to supply the sides. Numerous flame cells are visible scattered throughout the wall. Within the body

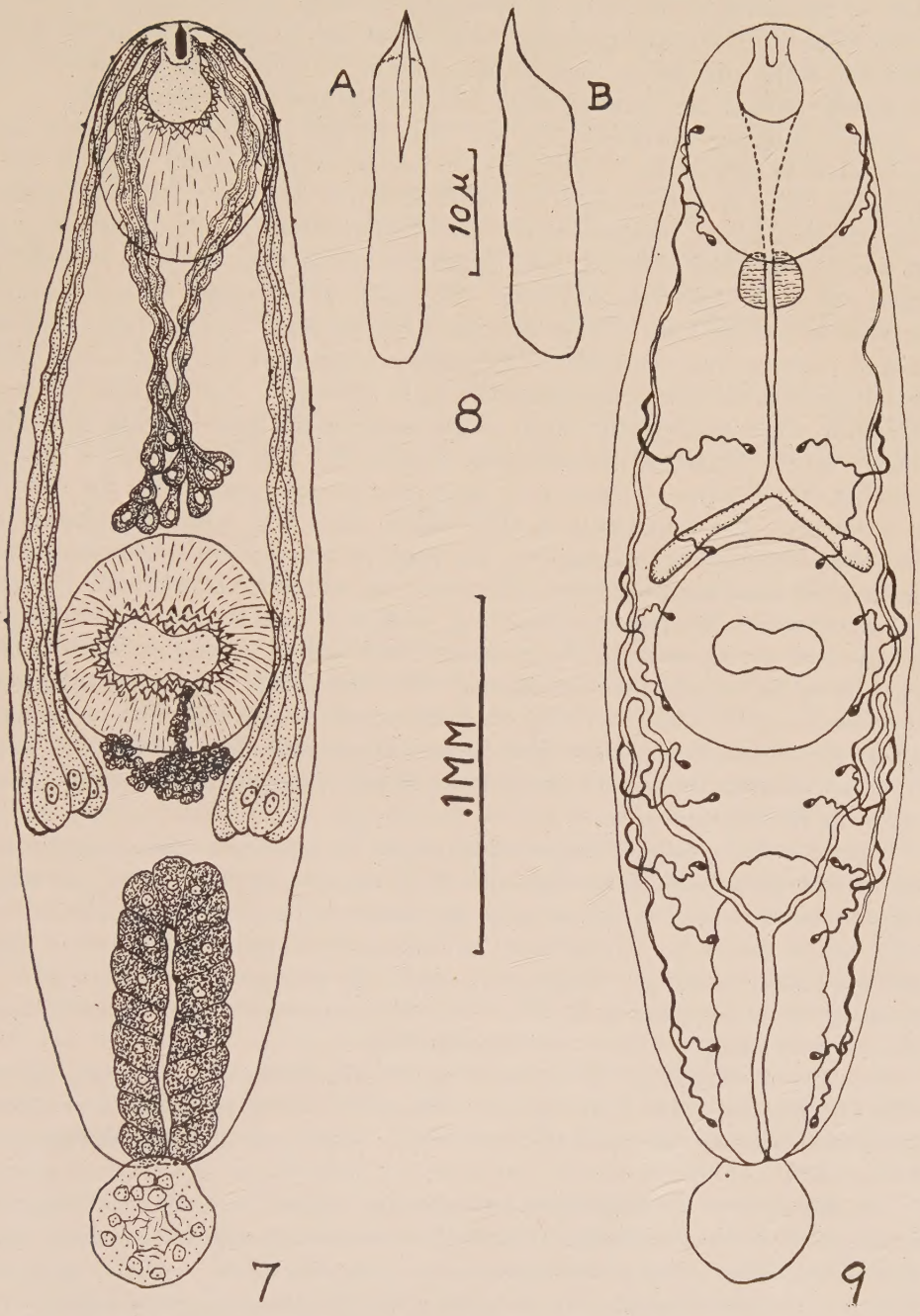

FIG. 7. Ventral view of cercaria showing morphological details.
FIG. 8. Stylet of cercaria. A—dorso-ventral view, B—lateral view.
FIG. 9. Excretory system of the cercaria. Freehand sketch on camera lucida outline.

cavity of the sporocyst the 20 to 40 developing cercarial embryos appear as ovoid bodies, all of which are approximately the same size and average 0.105 mm long by 0.080 mm wide. So far as could be determined, no increase in the number of developing cercariae had occurred since the initial division of the germ mass.

Mature daughter sporocysts (Fig. 6) when freshly expelled by the snails are pearly white in appearance. They are stout tubular structures with rounded ends, measuring 1.62–1.97 mm in length by 0.46–0.53 mm in diameter. The wall, which is covered by a cuticle, measures 0.045–0.105 mm in thickness. It consists of several layers of flattened cells with small nuclei and two or three layers of irregularly shaped cells with large rounded nuclei. The body cavity is lined by a membrane composed of a single layer of large flattened epithelial cells. Between the lining of the body cavity and the remainder of the sporocyst wall there is a tough noncellular layer, suggesting a cyst wall, approximately 1 μ in thickness. The cercariae are thus enclosed in a cyst-like structure lined on the inside by the layer of living epithelial cells and on the outside by the noncellular membrane. The name endocyst is proposed for this structure. There is no birth pore or canal penetrating the wall of the sporocyst. The body cavity is filled with a viscid fluid which was shown by *intra vitam* stains to be material from the large posterior glands of the cercariae. The 20 to 40 cercariae, all of which are mature, are suspended in this fluid.

The cercaria.—The cercaria (Fig. 7) is small, muscular, elongate oval in shape, and flattened dorso-ventrally. It measures 0.348–0.370 mm long by 0.095–0.105 mm wide in the region of the acetabulum. The thin cuticle, measuring 1–1.5 μ in thickness, is aspinose and smooth. In living specimens several pairs of sensory papillae are visible along the lateral margins of the anterior half of the body. The tail, which contains the nuclei of from 16 to 20 cells, is a short, rounded structure, measuring 28–38 μ diameter.

The muscular oral sucker is directed anteriorly but possesses a subterminal opening. It is oval in shape, measuring 0.059–0.063 mm long by 0.053–0.062 mm wide. The acetabulum, which is situated near the equator of the body, measures 0.064–0.071 mm in diameter. The exposed surfaces of both suckers are covered with very small scales or spines. Prepharynx absent; the pharynx is globular, muscular, and measures 20–21 μ long by 22–27 μ wide. The long slender esophagus bifurcates into two short ceca just anterior to the acetabulum.

The very small stylet (Fig. 8) measuring 26–30 μ in length, 5–7 μ in width, and 6–8 μ in depth, lies within a small pocket in the antero-dorsal wall of the oral sucker. This pocket communicates with the outside by a small opening in the anterior wall of the sucker.

On either side of the acetabulum and extending slightly posterior to it is a group of four large penetration glands. The ducts from these glands pass anteriorly by a slightly undulating course to the lateral walls of the oral sucker through which they open into the stylet pocket. The ducts on either side apparently fuse together and open into the stylet pocket through a common pore. Since these glands are detected with the greatest difficulty, it is likely that there are other dorsal to the acetabulum. Five pairs of smaller penetration glands occupy the midregion of the body between the oral sucker and acetabulum. The ducts from these glands, five on each side of the midline, pass anteriorly to open into the stylet pocket just medial to the large gland ducts.

The excretory pore is terminal and dorsal to the subterminally attached tail. The simple, tubular, I-shaped vesicle (Figs. 7, 9) which is composed of a layer of large dense cells, extends anteriorly about two-thirds the distance to the acetabulum. At its anterior end it receives a common collecting tubule from each side of the body. The two common collecting tubules pass anteriorly and laterally to about the equator of the acetabulum where each branches into an anterior and posterior main collecting tubule. The anterior main collecting tubules on each side pass forward to give rise to three accessory tubules, each of which branches into two capillaries. Similarly, the other two main collecting tubules pass posteriorly, giving rise to three accessory tubules, each of which branches into two capillaries. Each capillary tubule terminates in a single flame cell establishing a flame cell pattern of the type $2\ [(2+2+2)+(2+2+2)]$ for this cercaria.

In stained specimens the genital primordium appears as a deeply-stained mass of small cells lying in the midline just posterior to the acetabulum. From the main portion of the primordium a chain of cells extends anteriorly dorsal to the acetabulum.

DISCUSSION

The development of the larval stages of *E. procyonis,* as determined in experimentally infected snails, differs in several respects from that described for *Dicrocoelium dendriticum* by Mattes. Instead of producing a single generation of daughter sporocysts all of which mature simultaneously as reported for *D. dendriticum,* the mother sporocyst of *E. procyonis* produces daughter sporocysts continuously over a period of a month or more. The mature daughter sporocyst of *D. dendriticum* possesses a wall one cell layer thick except at the ends, and is provided with a birth canal. Cercariae develop and escape continuously from these sporocysts over a period of several months. In contrast, the mature daughter sporocyst of *E. procyonis* possesses a wall several cell layers thick, the inner of which is an epithelial membrane lining the body cavity. It has no birth canal and the cercariae which develop simultaneously from germ balls produced by the division of the germ mass in the immature form, remain confined in the sporocyst.

There is also a difference in the manner in which the cercariae of the two species leave their molluscan hosts and are protected after their departure. According to Neuhaus, the cercariae of *D. dendriticum* migrate out of the daughter sporocysts through the birth canal and actively penetrate into the mantle cavity of the snail. Here they collect in groups of 200 to 400 and form a common spherical cyst from the secretion of their large glands. These group cysts are forced to the respiratory opening by the breathing movements of the snail. Here from five to fifteen of these cysts adhere together in a large grape-like mass which becomes covered with mucus from the snail. As the snails crawl about, these masses are deposited on vegetation and other objects. In *E. procyonis,* where the cercariae are confined within the body cavities of the mature daughter sporocysts, the sporocysts themselves either migrate or rupture into the mantle cavity of the host where they collect in clumps. These masses of sporocysts, which become covered with mucus from the snail, are then expelled from the respiratory pore and are deposited on vegetation and other objects.

There is no difference in the fundamental morphology of the cercariae of *D. dendriticum* and *E. procyonis.* Other than the difference in the size and state of

degeneration of the tail, the two forms are strikingly similar in possessing a stylet, both large and small penetration glands, and a flame cell formula of 2 [(2+2+2)+(2+2+2)]. This similarity suggests that cercarial characters, particularly the flame cell formula, when they are known for other species of the family, will be useful in eradicating the taxonomic confusion existing within the DICROCOELIIDAE.

SUMMARY

Ova of *Eurytrema procyonis* Denton, 1942, when fed to the common garden snail, *Mesodon thyroidus* (Say), develop into mature mother sporocysts in 70 days. The mother sporocysts which develop in the haemocoelic space of the digestive gland are extensively lobed white masses. Within the matrix of the mother, daughter sporocysts are produced over a period of a month or more. The daughter sporocysts, after rupturing from the parent form, migrate to the respiratory portion of the mantle and surrounding tissues of the host where they mature in 141 days. Within the body cavity of the daughter sporocysts, a "fixed" number of cercariae are produced by the equal division into germ balls of the central mass of single germ cells present in them at an early stage of development. The daughter sporocysts, when mature, rupture into the mantle cavity of the host where they collect in clumps. These clumps of sporocysts are expelled from the respiratory pore by the movements of the snail and are deposited on vegetation and other objects. The styleted, almost tailless cercariae which have a flame cell formula of 2 [(2+2+2)+(2+2+2)] remain confined within the body cavity of the sporocyst. Whether or not a second intermediate host is involved and the mode of infection of the definitive host was not determined.

The larval forms of *E. procyonis* are compared with those of *Dicrocoelium dendriticum* (Rudolphi, 1819), the only other species of the subfamily DICROCOELIINAE Looss, 1899, for which they are known. The mother sporocyst of *E. procyonis* is similar in morphology to that of *D. dendriticum* but differs in its manner of producing daughter sporocysts. The daughter sporocysts of the two species differ both morphologically and in the manner and number of cercariae produced. The cercariae of *E. procyonis* and *D. dendriticum*, which have the same flame cell formula, are similar in their fundamental morphology.

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IXODES OZARKUS N. SP. AND *ORNITHODOROS AQUILAE* N. SP., WITH NOTES ON *O. TALAJE* AND *O. KELLEYI* (IXODOIDEA)*

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Three lots of a new species of *Ixodes* have been received from Dr. W. J. Baerg, University of Arkansas, Fayetteville, Arkansas. The accession numbers are as follows:

20760, Dog, Huntsville, Arkansas, Feb. 20, 1944, 3 females, 2 males; 20854, Deer, Huntsville, Arkansas, Feb. 20, 1944, 3 females, 2 males; 21023, Dog, Huntsville, Arkansas, March 1944, 3 females.

Dr. Baerg informed me that some specimens of this tick had also been taken from raccoon and that the tick readily attacks man. He further states, "When attached it is difficult to remove, and the effects on man seem to persist much longer than the effects of the common dog tick. This species of tick has been very active, getting on hunting dogs in large numbers during the months of December and January, as well as in February." The tick is described and figured below.

Ixodes ozarkus n. sp.
(Figs. 1 and 2)

Female

Body: Length (unengorged) 2.46 mm; width 1.65 mm. Oval, widest at about the middle. Color of well-sclerotized parts dark brown (nearly black). Scutum extending about half the length. Postscutal area finely and faintly striated, and with long fine hairs similar to those on the scutum. Marginal grooves distinct anteriorly, faint posteriorly. No fully fed females available for description.

Capitulum: Length 0.84 mm; width of basis (just posterior to palpi) 0.54. Basis with lateral profile lines mildly curved and converging posteriorly. Posterior margin nearly straight and with the salient edge slightly depressed over the distinct, small, pointed cornua. Porose areas large, depressed and separated by about the length of the shorter axis of one. Surface smooth, shining, faintly shagreened, impunctate and without hairs. Palpi long with articles 2 a little longer than 3. Combined length of 2 and 3, 0.69 mm. Greatest width 0.22 mm. Outer profile lines nearly straight, inner edges curved. Surface smooth, shining and with a few faint punctations. Hairs few and moderate in size.

In ventral view, basis is mildly constricted at the middle. Posterior margin broadly rounded, salient. Transverse sutural line distinct. Auriculae distinct as lateral saliences. Surface smooth, shagreened, impunctate and with a very few short hairs at the sides. Palpi flattened on their inner faces. Article 1 with an indistinct ventral plate.

Hypostome: Long, with sides approaching parallel, apex rounded. Denticles 3/3 for about three-fourths the length, then 2/2 to the base. Lateral denticles large, pointed; medians progressively smaller to the middle, rounded. Length 0.60 mm.

Legs: Long, smooth, faintly shagreened. Tarsi I especially long. Haller's organ noticeably distant from the distal end. Length of tarsus I, 0.71 mm; metatarsus, 0.48 mm. Length of tarsus IV, 0.66 mm; metatarsus, 0.48 mm.

Coxae: Coxae II, III and IV mildly convex, I, more convex. Coxa I with a long pointed inner spur, which overlaps II in unfed and little fed specimens. External spur on I very short and connected with the internal spur by a continuous edge which limits the posterior side of the coxa. External spurs on II, III and IV about the same as that on I. Coxal hairs long or short and few in number.

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*Contribution from Rocky Mountain Laboratory (Hamilton, Montana), Division of Infectious Diseases, National Institute of Health.

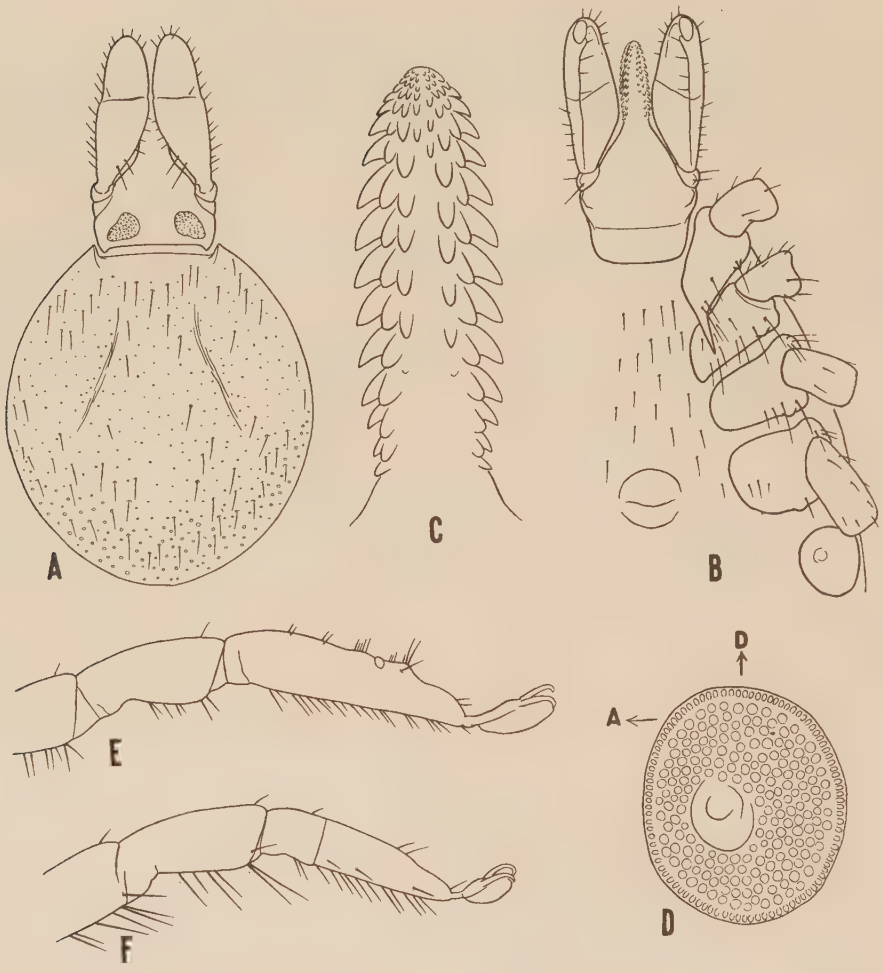

FIG. 1. *Ixodes ozarkus* n. sp., female. A. Capitulum and scutum, dorsal view. B. Capitulum and coxae, ventral view. C. Hypostome. D. Spiracular plate. E. Tarsus and metatarsus of leg I. F. Tarsus and metatarsus of leg IV.

Spiracular plate: Subcircular with the slightly longer axis transverse. Goblets moderate in size and number. Surface of plate nearly level. Length 0.39 mm, width 0.33 mm.

Sexual aperture: Situated between coxae IV.

Male

Body: Oval, widest at about the middle. Color as in the female. Length 2.40 mm, width 1.26 mm.

Capitulum: Dorsum of basis large, broad and flattened. Greatest width of basis 0.30 mm. Lateral profile lines nearly straight and converging posteriorly. Posterior margin straight or a little convex. Cornua absent. Lateral and posterior edges salient. Surface shagreened, punctate on the posterior half; and without hairs. Palpi short and broad. Articles 2 and 3 about equal in length, suture separating them depressed. Hairs for the most part short, but a few at the base are long.

In ventral view, basis is triangular with posterior margin a rounded salient point which is raised over the level of the "neck." Auriculae as small, rounded saliences. Palpi flattened on their inner faces. Article 1 visible as a ridge which is continuous with the posterior edge of 2 and 3 and has 2–3 hairs. Surface smooth, shagreened, impunctate and without hairs.

Hypostome: Short and broad and of the type found in *I. reduvius* (the genotype), *I. scapularis* and *I. pacificus*. Lateral denticles large and with the proximal pair especially large and

rounded; medians principally as transverse crenulations. Distal end of hypostome rounded or very faintly notched; basal portion free of denticles. Length about 0.26 mm.

Scutum: All peripheral areas about equally declivitous. Limits of pseudo-scutum not visible. Scapulae rounded. Lateral carinae absent. Cervical grooves faint, first convergent, then divergent; better seen in reflected light. Surface dulled by the distinct shagreening. Supine hairs numerous, long and fine, about evenly distributed; similar to those on the lateral folds. Puncta-

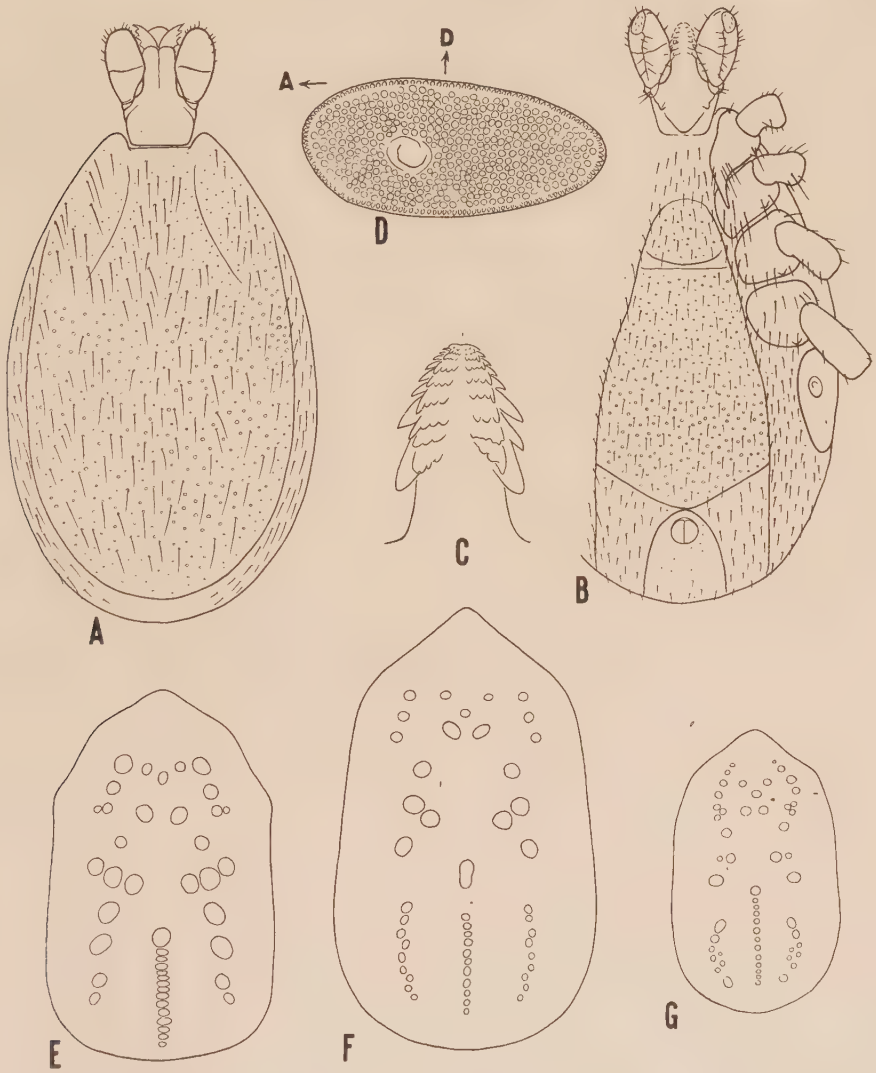

FIG. 2. *Ixodes ozarkus* n. sp., male. A. Capitulum and scutum, dorsal view. B. Capitulum, coxae and plates, ventral view. C. Hypostome. D. Spiracular plate. E. *Ornithodoros talaje,* female, showing shape, relative size, and pattern of discs. F. *Ornithodoros kelleyi,* female, showing shape, relative size, and pattern of discs. G. *Ornithodoros aquilae* n. sp., female, showing shape, relative size, and pattern of discs.

tions numerous, distinct and of moderate size, those in the central and lateral areas usually larger than those on the ends.

Ventral plates: Median plate about three times as long as the anal. Anal plate much wider behind, adanals much wider in front. Median and adanal plates with numerous distinct punctations; anal plate with only fine punctations. Hairs numerous and like those on the scutum excepting that they are fewer and shorter on the anal plate and on the posterior part of the adanals.

Legs and coxae: Essentially as in the female.

Spiracular plate: Oval, large, with longer axis longitudinal. Surface following the contour of the body wall. Goblets very numerous. Length 0.48 mm, width 0.24 mm.

Sexual aperture: Between coxae III.

Nymphs and larvae unknown.

Ixodes ozarkus resembles *I. scapularis,* which occurs in the same area, and *I. affinis,* which occurs in Central America and South America. *I. ozarkus* might easily be confused with *I. affinis* but in the latter the female scutum is more nearly circular and has conspicuous, large, deep punctations near the posterior margin. The female hypostome of *I. affinis* has four large files of denticles on each side, while *I. ozarkus* has only three. *I. affinis* has a distinct ventral plate on palpal article 1, and lacks the small cornua found in *I. ozarkus.* The male of *I. affinis* has larger, more distinct punctations on the median plate and has large, distinct punctations on the scutum which are limited to the central area. The female of *I. scapularis* is distinguished from the female *I. ozarkus* by having fewer hairs on the scutum, shorter files 3 of the denticles on the hypostome, and shorter spurs on coxae 1. The males of *I. scapularis* are smaller than in *I. ozarkus,* have the hypostome relatively narrow and the hairs on the dorsum shorter and less noticeable.

Holotype: Female from 20760.

Allotype: Male from 20760.

Paratypes: Females and males from 20760, 20854, and 21023.

Holotype, allotype, and paratypes are deposited in the Rocky Mountain Laboratory. Other types have been placed in the following: U. S. National Museum, Washington, D. C., paratype female and male; Department of Entomology, University of Arkansas, Fayetteville, Arkansas, paratype female and male; Division of Entomology and Economic Zoology, University of Minnesota, University Farm, St. Paul, Minn., paratypes female and male; Museum of Comparative Zoology, Harvard University, Cambridge, Mass., paratype female; Department of Entomology, University of California, Berkeley, Calif., paratype female.

Beginning in May 1943, Dr. R. B. Williams, Director, Wyoming Public Health Board, Cheyenne, Wyoming, sent us immature *Ornithodoros* ticks taken as larvae from the region of the head of raptorial birds. Some had molted into the first nymphal stage when received. These ticks were assigned accession numbers as follows:

20247, *Falco mexicanus,* Chalk Bluffs, Weld Co., Colorado, May 31, 1944, 12 larvae; 20262, *Falco mexicanus,* Chalk Bluffs, Weld Co., Colorado, Elev. 5780. June 16, 1943, 9 larvae; 20263, *Buteo regalis,* Chalk Bluffs, Weld Co., Colorado, June 16, 1943, 17 larvae; 20264, *Falco mexicanus,* Chalk Bluffs, Weld Co., Colorado, June 16, 1943, 6 larvae; 20267, *Aquila chrysaetos canadensis,* Simpson Creek, Weld Co., Colorado, June 19, 1943, many larvae; 20269, *Falco mexicanus,* Pawnee Buttes, Weld Co., Colorado, June 20, 1943, many larvae; 20270, *Aquila canadensis,* Big Simpson Creek, Weld Co., Colorado, June 20, 1943, many larvae; 20275, *Aquila chrysaetos canadensis,* near Chugwater, Platte Co., Wyoming, June 26, 1943, 13 larvae; 20278, *Aquila chrysaetos canadensis,* Big Simpson Creek, Weld Co., Colorado, July 4, 1943, 27 nymphs; 20279, *Aquila chrysaetos canadensis,* Chalk Bluffs, Weld Co., Colorado, July 3, 1943, 18 nymphs; 20280, *Falco sparverius sparverius,* Big Simpson Creek, Weld Co., Colorado, July 3, 1943, 1 nymph; 20281, *Falco mexicanus,* Chalk Bluffs, Weld Co., Colorado, July 4, 1943, 11 nymphs.

The larvae were first fed on a hawk, and later on mice and guinea pigs. Attempts to feed them on a young chicken were not successful. Some of the larvae were reared to the adult stage and five females and four males were obtained.

From larval, nymphal, and especially from adult characters, the ticks were found to be a new species which is described and figured below.

Ornithodoros aquilae n. sp.
(Figs. 2 and 3)

Adult

Body: Oval with sides nearly parallel, and with excavations near legs I and IV variable (usually less pronounced than in either *O. kelleyi* or *O. talaje*). Anterior margin extended into a rounded deflexed point in front. Size of female: largest of five, 5.10 long by 2.49 wide; smallest of five 3.90 by 1.95 wide; average length and width 4.53 by 2.49. Size of male: largest of four, 3.90 long by 1.92 wide; smallest of four 3.48 long by 1.65 wide; average length and width 3.74 by 1.83.

Mammillae: On dorsal surface the mammillae are large, placed close together but not crowded; about equal in size in median and peripheral areas, distinctly larger in posterior than in anterior areas. The best defined individual mammillae show definite ridges on the sides which radiate from top to base; many have each an imperfect depression at the top which continues as a groove extending to near the base. On the venter, the mammillae in the peripheral areas are as large as those on the dorsum with those in the median areas becoming progressively smaller and less definite to the middle, excepting that those on the supracoxal folds are smallest and least definite.

Discs: On dorsal surface, large, conspicuous and with their immediate edges raised, those in the median area separated by about two to four staggered rows of mammillae. On ventral surface, discs are limited to the paired preanal and the median postanal grooves in which they are in lineal arrangement and present also in three groups caudad of the transverse postanal groove.

Legs: Small and of moderate length; surface micromammillated; hairs small. Length of female tarsus I, 0.6; metatarsus, 0.48. Length of female tarsus IV, 0.72; metatarsus, 0.6.

Coxae: Coxae I and II separated, all others contiguous. Surfaces partly micromammillated and partly with excrescences.

Hood: Not much in evidence, merging into the anterior extensions of the supracoxal folds, anterior end terminated by the deflexed, rounded anterior point of the body wall.

Cheeks: Large, suboval, and when apposed largely covering the palpi and hypostome. Both ends free. Surface very irregular.

Capitulum: Quadrangulate, much wider than long, surface micromammillated and with transverse wrinkles; hairs absent excepting 1 pair well back of hypostome and in addition to the usual posthypostomal hairs. Papal article 1 micromammillated.

Hypostome: Short and small, sides nearly parallel, mildly notched apically. Denticles with two short files of large denticles on each side, and with a third pair of very small denticles which are lacking in *O. talaje* and *O. kelleyi*. Length about 0.32 (female), 0.20 (male).

Folds: Coxal and supracoxal folds present, the latter extending anteriorly into the faint hood.

Grooves: Preanal, transverse postanal and median postanal grooves present. Dorso-ventral grooves absent.

Sexual aperture: At the level of the intervals between coxae I and II in the female; between coxae I in the male.

Eyes: Absent.

Nymph

As in most species of *Ornithodoros,* late stage nymphs of this species differ from the adults principally in size and absence of the sexual aperture.

Larva

Unfed larva suboval; basis capituli triangular in dorsal view. Palpi very long, slender and in life appressed against the hypostome. Average length of body including the capitulum 0.64.

Hypostome: Long and slender, but less slender than in *O. kelleyi;* denticles 3/3 for about the apical one-third, then 2/2 to the base; medians much less visible than the laterals even in specimens mounted in balsam. Length, apex to basal denticles, 0.64.

Holotype: Reared female from 20278.
Allotype: Reared male from 20278.
Paratypes: Adults reared from Nos. 20247, 20262, 20263, 20270, 20278.
Paratype larvae from 20270.

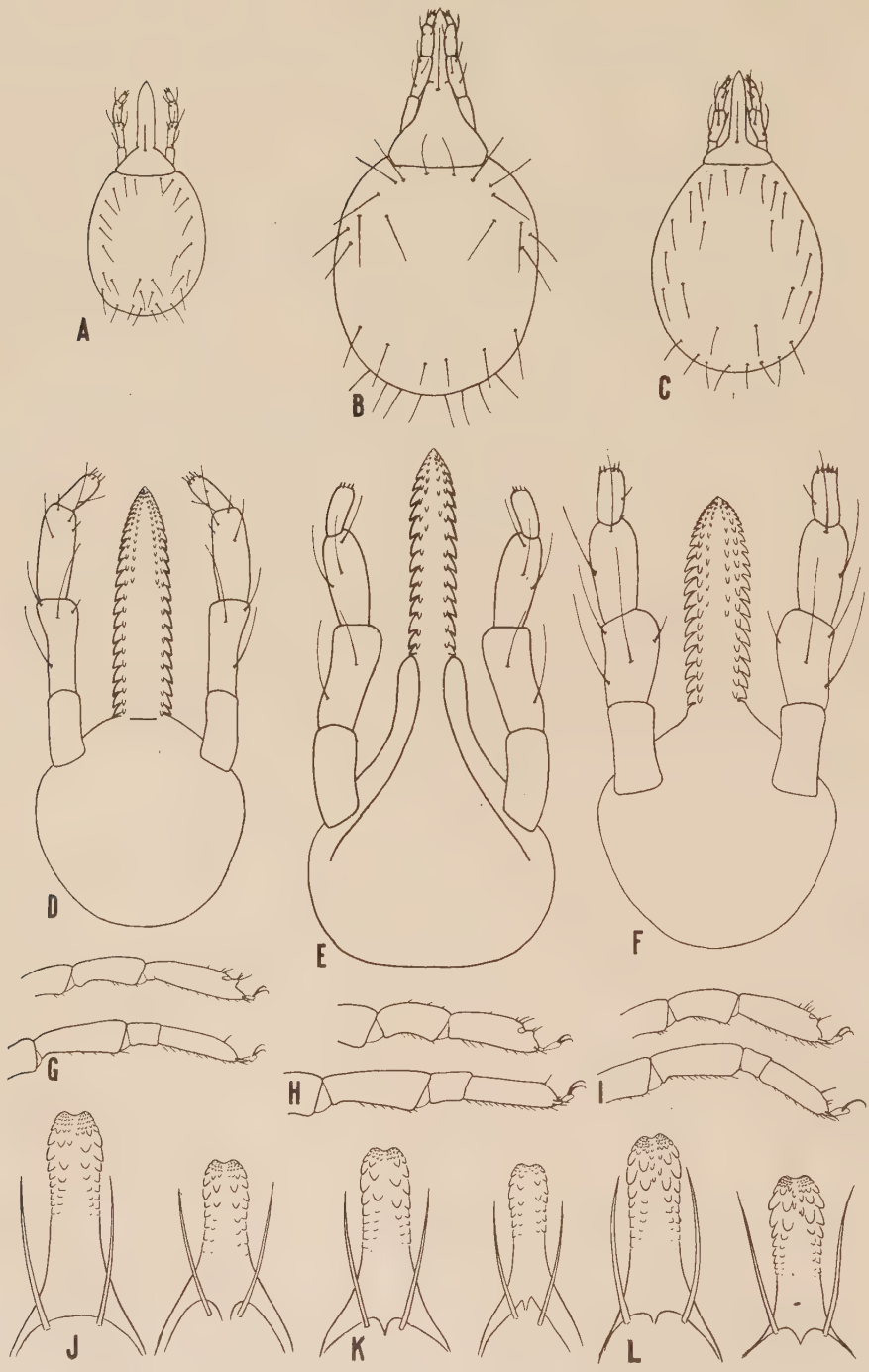

FIG. 3. Each part shown in relative size. A. *O. talaje* larva, capitulum and body, dorsal view. B. Same of *O. kelleyi.* C. Same of *O. aquilae.* D. *O. talaje* larva, capitulum, ventral view. E. Same of *O. kelleyi.* F. Same of *O. aquilae.* G. *O. talaje,* female, tarsus and metatarsus of legs I and IV. H. Same of *O. kelleyi.* I. Same of *O. aquilae.* J. *O. talaje,* hypostome of female (on left) and male. K. Same of *O. kelleyi.* L. Same of *O. aquilae.*

Holotype, female, allotype, male, and paratype larvae deposited in the collection of the Rocky Mountain Laboratory.

Paratypes male, female, and larvae deposited in the U. S. National Museum, Washington, D. C., the Museum of Comparative Zoology, Harvard University, Cambridge, Mass., and the Division of Entomology and Economic Zoology, University of Minnesota, University Farm, St. Paul, Minnesota.

O. aquillae, O. talaje, AND *O. kelleyi* COMPARED

O. aquilae and *O. kelleyi* Cooley and Kohls, 1941, are both closely related to *O. talaje* (Guérin-Méneville, 1849) and could easily be confused with it. Species that have been incorrectly identified as *O. talaje* in the past and have been so referred to in the literature are: *O. hermsi, O. rudis, O. coprophilus* (now *Antricola coprophilus*), and *O. migonei.* This confusion resulted from the inadequacy of the early descriptions of *O. talaje.*

"*O. talaje*" in past years has been reported from Venezuela, Mexico, New Granada, Santiago de Chile, Colombia, Hawaii, Florida, Texas, and California, and in view of recent findings it becomes apparent that further studies may show that some of the above locality records have been in error.

Following are comparisons, descriptions, and figures of *O. aquilae* with *O. talaje* and *O. kelleyi.* The descriptions and figures of *O. talaje* are based on specimens of all stages collected near the type locality in Guatemala. I am grateful to Dr. E. Brumpt of Paris for this material, which includes adults and reared material.

The descriptions and figures of *O. kelleyi* are based on all the materials in the collection of the Rocky Mountain Laboratory, including the types and specimens subsequently collected.

All drawings of *O. aquilae, O. talaje* and *O. kelleyi* in figures 2 and 3 were made to conform to the average measurements, so that the figures show relative size.

In describing *O. talaje,* Guérin-Méneville (1849) quotes from notes of M. Nicolet as follows (translated by Virginia Graybeal):

> At Casa Vieja de Gastoya, road from Guatemala to Zacopa (Central America) 15 leagues from Guatemala, May 5, 1847, I was awakened several times in the midst of very profound sleep, by some excruciating bites on my hands and body, and my companion, M. Jules, complained even more than I. At 3 o'clock, irritated by these painful bites, I lighted a candle, and found my hands covered with blood and some spots resembling large lice-bites, which I believed must belong to a particularly large species. My companion told me that we might have been bitten by some wasps lodged in the wall of the house. Having awakened the "arriero" (muleteer) who conducted us, and having told him what we had found, he informed us that we were victims of an animal named "Talaje" which was regarded as a large bug. I then relighted my candle in order to look for this insect, and soon I found this *Argas,* which was nauseating to me. Some were filled with blood, and others had a wrinkled skin. I then recalled having found some on my body during the night and rolling them between my fingers, taking them for some of the ticks with which my mule was covered (au point que) several persons told me that it might die of it.
>
> I took a certain number of these *Argas,* which I put in a tube in order to study them in Paris.
>
> These Talajes inhabit cracks in the walls of old houses. These walls were made of rough bamboo and plugging-mortar. They bite in the manner of bugs, and return to their holes during the day for they are nocturnal.

Nuttall (1908) states, "Habitat: native houses, attacks man."

From the above it is apparent that man is the only known host of *O. talaje* in the type locality.

Measurements of available adults were as follows: largest of six females, 6.00 × 4.40; smallest, 4.95 × 3.70. Average, 5.54 × 3.72. One available male 4.85 × 2.75. Average length of many larvae 0.64.

In the adults of *O. talaje* the mammillae are very large in all peripheral areas. The dorsal surface is occupied very largely by the depressed areas which include the large discs, and in which the mammillae are few in numbers. The adult hypostome is very much like that of *O. kelleyi* and differs from both *O. kelleyi* and *O. aquilae* in lacking the short 3d file (median) of denticles.

The larvae of *O. talaje* are the smallest of the three species here compared and have an average measurement of 0.66. The hypostome lacks the anterior projection from which the denticled portion continues.

O. kelleyi. This species was originally described from three nymphs taken from bats, and from two cast larvae skins, taken from near the Utah-Colorado State line. Later studies were made of numerous specimens of nymphs and adults taken from houses and other buildings in New York, Wisconsin, Minnesota, Pennsylvania, Illinois, and Iowa—all northern states. All of these buildings were reported to be frequented by bats. Larval studies are from material kindly furnished by Dr. W. A. Riley, which had hatched from eggs laid by a female taken from a house in Minnesota.

The only known hosts of *O. kelleyi* are bats.

Measurements of all available adults are as follows: largest of six females 7.50 × 4.00; smallest, 5.97 × 3.24. Average 6.83 × 4.00.

The average length of the larva of *O. kelleyi* is 1.09.

Larval hypostome long, very narrow, sides parallel, placed on an anterior extension of the basis. Length of portion with denticles 0.18; length from insertion of the palpi to apex, 0.30.

SUMMARY OF COMPARISON OF *O. talaje, O. kelleyi* AND *O. aquilae*

Adults of *O. kelleyi* are largest, *O. talaje* intermediate, and *O. aquilae* smallest. Mammillae are relatively largest in *O. talaje* and distinctly fewer in number because the discs and the depressed areas which include them are relatively larger.

The three species show only minor distinctions in tarsus I. That of *O. talaje* is longer and more slender than in *O. aquilae*. Tarsus IV of *O. kelleyi* is distinctly longer than tarsus I. The hypostomes are all very much alike excepting that in *O. aquilae* both sexes show files 3 present.

The larvae of *O. aquilae* are intermediate in size between *O. talaje* and *O. kelleyi; O. kelleyi* is largest. The larval hypostomes of *O. aquilae* and *O. talaje* are very much alike but lack the anterior extension from which the barbed portion continues, which is present in *O. kelleyi.*

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THE RESPIRATION OF THE PROTOZOAN PARASITE, *EIMERIA TENELLA*[1]

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INTRODUCTION

The economic importance of the protozoan parasite, *Eimeria tenella,* has stimulated a great deal of research along the lines of pathology, specificity, prevention, and its resistance to environmental factors such as temperature and chemicals. Certain of the more academic studies of this parasite have been somewhat neglected. The authors are unaware of research which measured the metabolic rate of the parasite itself or of the tissues invaded by the parasite. It was to supplement our knowledge along this line that work on the metabolic rate of the parasite and invaded tissues was undertaken.

MATERIALS AND METHODS

Source of the oöcysts and infected tissues.—Single Comb White Leghorn chickens supplied by the Department of Poultry Husbandry of the University of Wisconsin were used to supply the oöcysts and tissues for this investigation. Those used to produce oöcysts weighed approximately 700 grams, while those used in studies on the respiration of the coccidia within the epithelial cells of the cecal pouches weighed approximately 300 grams. All chickens were inoculated with approximately 50,000 sporulated oöcysts and were placed in sterile wire cages with outside feed and water containers at the time of infection.

Preparation of unsporulated oöcysts. On the 9th day after infection the birds were sacrificed and the cecal pouches removed. The cores and scrapings of the cecal pouches containing the oöcysts were placed in the Waring blender with sufficient water to just cover the blades. From 3 to 5 minutes were found to be sufficient to separate the oöcysts from the cecal material.

At the end of the mixing period a sufficient amount of water was added to increase the volume about 3 times. The resulting mixture was placed in a large separatory funnel and allowed to stand for 30 minutes. The sediment was then transferred to 50-cc centrifuge tubes.

The method of concentrating the oöcysts and washing them free of bacteria was a modification of that used by Lane (1922) for concentrating helminth eggs and by McCoy (1929) for the sterilization of hookworm eggs. The sediment was washed clear of much of the extraneous material by repeatedly centrifuging with top water at a speed of about 1000 revolutions per minute. When the supernatant fluid became clear, saturated sodium chloride solution was added to the sediment and the

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tubes again centrifuged. The resulting surface film containing the oöcysts was transferred to another centrifuge tube by means of a piece of glass tubing or the open end of a small vial. The transferred oöcysts were centrifuged again with sterile distilled water to free them of the concentrated salt solution.

Bacteriological counts of the oöcyst suspension after repeated washing and flotation with salt solution revealed only a small number of bacteria. To free the suspension of the few remaining bacteria, 5 per cent antiformin made up in 10 per cent formalin was added and the mixture allowed to stand for 5 minutes. The oöcysts were again concentrated by centrifuging and the antiformin-formalin solution poured off. To test the sterility of the suspension, 0.1 cc was inoculated into tubes of nutrient agar, which were incubated at 37° C for 24 hours. Only the respiratory determinations on the bacteria-free oöcyst suspensions are included in this report. The time required to prepare the oöcysts and the respiratory apparatus for the initial reading was approximately 4 hours.

The preparation of sporulated oöcysts.—The oöcysts to be sporulated were freed from the host tissue by means of the *Waring blender* as described above, and were then placed in a thin layer in Petri dishes and diluted about 5 times with 2.5 per cent potassium dichromate. The Petri dishes were left partially open to maintain adequate oxygen in the solution.

The procedure followed in preparing the sporulated oöcysts for respiratory determinations was much the same as that described for unsporulated oöcysts. The sporulated oöcyst suspension was passed through a 100-mesh copper sieve to remove larger particles. The filtrate was centrifuged several times in water to remove the potassium dichromate solution and most of the extraneous material. Saturated sodium chloride solution was used for oöcyst flotation and 5 per cent antiformin made up in 10 per cent formalin solution was used as described above to kill the bacteria.

Method of counting the oöcysts.—To count the oöcysts, 0.1 cc of oöcyst suspension was diluted with 0.9 cc of water. After thorough mixing a drop of the suspension was placed on a Spencer Bright-Line hemacytometer. The öocysts in 5 of the millimeter squares were counted and the number multiplied by 2 to get the number for 1 cubic millimeter. The final values were all expressed in cubic centimeters.

The respiratory apparatus, method of calculation, and solutions.—The Warburg apparatus was used throughout this investigation for respiratory determinations. The method of calculating the data was according to that of Dixon (1934). To calculate the amount of oxygen absorbed in the respiratory flasks from the manometer readings, it was necessary to know the manometric constant for each of the flasks except that used as the thermobarometric flask. These constants were calculated by substituting the proper values in the following equation and simplifying:

$$Ko_2 = \frac{V_g \frac{273}{T} \text{ plus } V_f a}{P_o}$$

where V_g is the volume of gas between the surface of the liquid of the flask and the 150-millimeter mark on the manometer, V_f is the volume of the liquid within the flask, T is the absolute temperature of the water bath, and P_o is the pressure of the Brodie solution of the manometers. Once the constant (Ko_2) for a flask

at a given temperature was known, the rate of oxygen consumption could be readily calculated by use of the following formulas:

$$\text{for oöcyst suspensions: } Qo_2 = \frac{hKo_2 \times 10^6}{\text{number of oöcysts}}$$

$$\text{for tissue respiration: } Qo_2 = \frac{hKo_2}{\text{dry weight of tissue}}$$

where h is the reading of the manometer in cubic millimeters.

Phosphate buffers were used as the substrate in most of the experiments and the different pH intervals prepared by mixing in different proportions, 0.1 M potassium monobasic phosphate and 0.1 M potassium dibasic phosphate. For pH ranges below 4.5, KCl–HCl buffers and phthalate buffers were prepared according to the method described by Clark in his book on the determination of hydrogen-ion concentration.

PRESENTATION OF DATA

The respiration of cecal pouch tissue parasitized by E. tenella.—To determine the respiratory rate of normal tissues and that of tissues parasitized by *Eimeria,*

TABLE 1.—*Showing the difference between the O_2 consumption of normal and parasitized cecal pouch tissue. (Qo_2 = cmm O_2 per mgm of tissue per hour)*

Experiment No.	Normal cecal pouch tissue			Parasitized cecal pouch tissue		
	Flask No.	Qo_2	Wt. of tissue in mgm	Flask No.	Qo_2	Wt. of tissue in mgm
1	2	7.5	4.8	4	10.7	3.4
	3	5.8	5.4	6	6.6	4.4
2	1	4.8	3.0	4	7.8	4.0
	2	3.7	4.0	6	4.3	3.9
				7	6.0	3.1
3	1	3.2	6.0	4	10.4	6.7
	2	5.5	4.4	6	7.0	6.3
4	1	3.3	6.3	3	7.9	5.2
	2	5.5	11.0	6	8.7	5.0
				7	7.4	6.0
5	1	4.8	4.5	4	7.9	4.0
	2	3.7	3.0	6	4.3	3.9
				7	6.0	3.1
Mean		4.7 ± .08			7.3 ± .11	

small, relatively uniform samples were removed from the cecal pouches of the chickens by means of a tonsil punch, 4 millimeters in diameter. The tissue samples were removed as quickly as possible (a few seconds after the chickens were killed), washed in sterile saline, then in a 1 to 1 mixture of a phosphate buffer (pH 7.3) and 1–500 solution of sulfanilamide. Two cc of the same solution were placed in the respiratory flasks to serve as substrate. The temperature of the flasks was maintained at 38° C and readings of the manometers were taken at 30-minute intervals over a period of 2 hours. At the termination of the experiment the tissues were removed to a weighing bottle and reduced to a constant weight in a drying oven. The dry weights were used to calculate the oxygen consumption and all rates were expressed in terms of oxygen consumed per milligram of the dry tissue per hour. Two Warburg flasks containing normal tissues and three containing parasitized tissue were run simultaneously.

The results of experiments to determine the respiratory rate of normal and parasitized tissue are shown in Table 1. The mean Qo_2 for the 10 determinations on

normal cecal pouch tissue was 4.7, and that for the 13 determinations on parasitized tissue was 7.3. The data on the oxygen consumption of both types of tissues were analyzed statistically and found to be significantly different. It must be concluded, therefore, that the parasites had a significant effect on the oxygen consumption of the cecal pouch tissue.

Normal respiration of oöcysts.—Normal respiration in this case is that of oöcysts placed under conditions known to be satisfactory for practical laboratory work. When oöcysts are kept at room temperature or slightly above (30° C) and at a pH of approximately 7.3, they sporulate rapidly and uniformly, and may then remain viable for over two years. The temperature of 30° C and pH 7.3 were taken as the normal. The number of oöcysts in each Warburg flask varied among the different experiments and ranged from 3,000,000 to 12,000,000 per cc. The number of oöcysts per flask was the same in any one experiment and the calculations were all reduced to the amount of oxygen per million oöcysts. Determinations were made for varying periods with readings taken at half-hour intervals.

TABLE 2.—*The rate of oxygen consumption of unsporulated oöcysts during the first twelve hours.* (Qo_2 = *cmm* O_2 *per million oöcysts per hour*)

Exp. No.	Flask No.	1st hr.	2nd hr.	3rd hr.	4th hr.	5th hr.	6th hr.	7th hr.	8th hr.	9th hr.	10th hr.	11th hr.	12th hr.	Number of oöcysts
2	1	12.2	10.0	13.0	11.2	9.0	12.0	11.0	8.0	10.0	14.0	20 2	26.0	3,560,000
4	3	20.8	12.7	17.3	15.0	17.3	17.3	15.0	16.1	13.8	23.1	27.7	39.3	3,000,000
4	6	14.5	15.3	16.6	15.0	16.6	15.3	29.3	8.3	14.0	15.3	35.3	38.9	4,000,000
28	1	8.0	6.9	10.2	7.0	7.1	7.2	6.9	6.8	8.0	10.0	18.9	31.0	2,000,000
30	1	18.9	9.4	18.9	14.2	17.8	16.6	14.2	14.2	14.2	13.0	27.3	37.9	2,000,000
30	2	19.2	13.2	18.0	13.8	16.2	18.0	15.6	14.4	14.4	13.2	26.4	38.4	2,000,000
Average		15.6	11.2	16.6	12.7	14.0	14.4	15.3	11.3	12.4	14.7	26.0	35.2	

Data on the unsporulated oöcysts are presented in Table 2 and show that little change in Qo_2 occurred during the first 10 hours. There was however, a great change following this period. The Qo_2 for the first 10 hours ranged from 11.3 to 15.6. During the 11th hour the Qo_2 increased to 26.0 and on the 12th hour was 35.2. This increase is in accord with the findings of George Ott (unpublished data) who found that there was little visible change in the protoplasm of the oöcysts during the first 12 hours, but a marked change during the second 12-hour period. Circumstances beyond control prevented the completion of the Qo_2 determinations between the 12- and 48-hour period. By the time the oöcysts were 48 hours old, the Qo_2 had decreased to about 0.4.

There was considerable variation in the Qo_2 of the various batches of oöcysts but when the data were examined statistically the variation was not significant.

The determinations on the normal rate of respiration of sporulated oöcysts were performed under conditions similar to those for unsporulated oöcysts. The temperature of the water bath of the respirometer was 30° C, the pH of the substrate was 7.3, and the length of the determinations was 3 hours. The concentrations of the oöcyst suspensions used were from 5 to 12 millions. The results of these determinations are given in Table 3. An inspection of the Qo_2 values shown in this table will reveal the marked difference between unsporulatd and sporulated oöcysts as to rates of respiration.

The number of oöcysts used in the 23 determinations on sporulated oöcysts may

TABLE 3.—*The rate of O_2 consumption of sporulated oöcysts over a period of three hours. (Qo_2 = cmm O_2 per million oöcysts per hour)*

Exp. No.	Flask No.	Qo_2	No. of oöcysts
21	6	1.00	12,420,000
20	3	0.60	12,300,000
18	1	0.61	11,500,000
14	2	0.23	10,000,000
14a	3	0.16	10,000,000
14b	4	0.23	10,000,000
14c	6	0.33	10,000,000
14d	1	0.20	10,000,000
13	1	0.30	7,920,000
13a	2	0.50	7,920,000
13b	3	0.60	7,920,000
13c	4	0.40	7,920,000
13d	6	0.40	7,920,000
13e	7	0.50	7,920,000
11	1	1.30	7,330,000
10	1	0.38	7,000,000
10a	2	0.56	7,000,000
15	1	0.33	7,000,000
15a	2	0.36	7,000,000
15b	3	0.46	7,000,000
12	1	0.90	6,000,000
16	1	0.26	5,670,000
16a	3	0.46	5,670,000

be considered in 4 classes: (1) 6 to 7 millions, (2) 8 millions, (3) 10 millions, (4) 11 to 12 millions. The average Qo_2 for the 4 classes in their order were 0.5, 0.4, 0.2, and 0.7. These values show on inspection a decrease in oxygen uptake as the number of oöcysts used increased from 6 to 10 million, but above 10 million the rate increased over all other values. Statistical analysis was made of values in two of the number classes to determine the significance of the difference between the mean values of the groups. The two classes selected were the 8 million (Exp. 13) and the 10 million class (Exp. 14). The mean rate for the 10 million class was 0.3 mm^3 per million oöcysts per hour, and the standard deviation was 0.15 With the 8 million the mean was 0.8 and the standard deviation 0.11. When these values were substituted in the proper statistical formulas, no significant difference was revealed between the 2 means. The oxygen uptake of the sporulated oöcysts is so small, and the variations between rates taken at different times is so great that it is probable that a much larger number of determinations will have to be made before any distinct trend in uptake due to numbers can be detected. Some of the possible reasons for the variations seen between runs will be discussed later.

The effect of pH on oxygen consumption of oöcysts.—Six experiments were conducted to determine the effect of a given pH range on the oxygen consumption of sporulated oöcysts. Each of the experiments consisted of 3 flasks with 2 cc of phosphate buffer and 1 cc of an oöcyst suspension. The pH intervals used throughout the series were 4.7, 7.3, and 8.8. The method of preparation of buffers at the various pH intervals was according to that described under the section on

TABLE 4.—*The effect of pH on the Qo_2 of sporulated oöcysts of* Eimeria tenella. *(Qo_2 = cmm of O_2 per million oöcysts per hour)*

Exp. No.	No. of hours observation	Rate of O_2 consumption in phosphate buffer at different pH intervals			No. of oöcysts used
		pH 4.7	pH 7.4	pH 8.8	
17	6	0.24	0.22	0.22	7,000,000
18	6	0.72	0.64	0.71	11,500,000
19	6	0.82	0.80	0.93	16,000,000
20	6	0.22	0.20	0.20	12,300,000
21	6	0.96	0.92	0.90	12,420,000
22	6	0.43	0.44	0.40	18,000,000

materials and methods. Each of the experiments ran for 6 hours and the Qo_2 values, which are given in Table 4 represent averages of the rates of oxygen consumption for the total length of each experimental run.

Because of the large amount of variation in rates of oxygen uptake between determinations made at different times and on different oöcyst suspensions, any conclusions drawn as to the effect of pH on the rates of oxygen consumption must be based on comparisons of the values within each experiment. Some of the differences may be due to differences in the number of oöcysts in the various experiments and some may be due to some physiological differences in the oöcysts themselves at the time of the experiments. In spite of these differences, the outstanding fact is that the pH values within the range considered had no significant effect on the rate of respiration of sporulated oöcysts.

Since sporulated oöcysts exhibited such a high resistance to the effects of pH, a characteristic resistance which these organisms show toward other chemical factors, it was surmised that a different type of response might be produced with unsporulated oöcysts. To test this hypothesis an experiment was performed with

TABLE 5.—*The effect of pH on the respiration of unsporulated oöcysts*

Flask No.	Type of substrate	pH of substrate	Rate of O_2 consumption in cmm/10^6/hr.	Number of oöcysts
1	KCl–HCl	1.3	16.0	2,000,000
2	Phthalate	2.4	16.4	2,000,000
3	PO_4–buffer	4.7	15.6	2,000,000
4	PO_4–buffer	7.3	15.3	2,000,000
5	PO_4–buffer	8.8	15.6	2,000,000

unsporulated oöcysts in substrates at 5 different pH intervals. The substrates used were KCl–HCl buffer at pH 1.3, phthalate buffer at pH 2.4, and phosphate buffers at pH 4.7, 7.3, and 8.8. The data (Table 5) reveal that differences in rates are too small to be attributed to differences in pH of the substrates. On the basis of all experiments presented it must be concluded that pH of the substrate within the limits tested has no effect on the respiration of oöcysts which can be detected by the methods used in this investigation.

DISCUSSION

Respiration of normal and parasitized tissue.—When the epithelium of the cecal pouches of chickens infected with *Eimeria tenella* is examined it is not surprising to find a significant increase in the respiratory rate of the parasitized tissue. The parasitized cells are greatly enlarged; the submucosa is packed in many areas with displaced epithelial cells and the small capillaries normally present have become greatly distended with blood. The general picture is that of an inflammatory reaction. While it is not difficult to imagine an increase in metabolism of the parasitized tissue, it is not easy to explain just how much of the increase is due directly to the parasites themselves and to the increase due to the pathology of the tissues. It was not the purpose of this study to determine the cause of the increase but rather to measure the change in the metabolic rate of the parasitized cells if a change did occur.

Since the tissues studied were not completely freed of bacteria, the effect of these organisms on the results of the experiments must be considered. The possibility

that the bacteria were responsible for the difference in the metabolic rate of the parasitized and normal tissues seems unlikely. The tissues were prepared in exactly the same manner, and George Ott (unpublished data) found no significant change in the number of types of bacteria present during the development of coccidiosis.

Respiration of sporulated and of unsporulated oöcysts.—In the unsporulated oöcysts there is a rounded mass of undifferentiated protoplasm. During the first 24 hours following discharge of the oöcysts into a favorable external environment, the oöcyst undergoes a division into 4 spores each containing 2 sporozoites. Within 48 hours practically all of the visible oöcysts have undergone complete development and have entered the resting stage with a resulting greatly reduced metabolic rate.

When the data on the metabolism of the 2 stages of oöcysts are examined the difference in Qo_2 is obvious. It is also evident that there existed a wide variation in the metabolic rate of oöcysts produced by different groups of chickens. No attempt will be made to explain this variation. That point alone may require years of concentrated study to solve. There are, however, several factors which can be mentioned that may throw some light on the subject.

The oöcysts are the product of the second generation merozoites which are released on the 5th day following infection. With this release there is a sudden and great decrease in the number of erythrocytes due to hemorrhage (Herrick, Ott, and Holmes, 1936). There is also a drop in hemoglobin, a great decrease in non-protein and total nitrogen, and concomitant with these changes, there is a great increase in blood sugar (Pratt, 1940, and Waxler, 1941) and blood chlorides (Waxler, 1941). It is during the 6th and 7th day following infection that the temperature may drop several degrees and the greatest mortality occurs. The mortality from coccidiosis may reach 100 per cent but that is unusual even from heavy artificial infections. In nearly every infection some chickens recover. It is only those that pass through the various pathological changes and recover that ever produce oöcysts. It is conceivable, therefore, that any one of the factors mentioned may affect the metabolism of the resulting oöcysts. It has been discovered by S. A. Edgar (to be published soon) that late on the 6th day, the macrogametocytes store relatively large quantities of glycogen. Undoubtedly other materials are stored at this time for use in the later sporulation process. It has been shown that canaries infected with malaria are more severely affected if fed sugar (Hegner and MacDougall, 1926), and rats infected with some pathogenic trypanosomes survive longer if starved or injected with insulin than if given normal treatment and feed. It is also reasonable to think that chickens which exhibit a greater resistance to coccidiosis may greatly affect the physiology of the oöcysts produced. It is conceivable that the amount of blood sugar in the blood of the chicken, the nitrogen level, or the amount of hemoglobin in the blood could affect the amount or quantity of materials stored by the gametocyte and thus affect their metabolic rate during subsequent development.

It is soon learned by anyone working with coccidian oöcysts that certain batches remain viable for long periods, two years or more, while others lose their infectivity within a few months. Whatever factor or factors affect the viability of the oöcysts may also cause the variation in metabolism observed among oöcysts secured from different chickens. Such explanation must await future work.

The effect of pH on respiration.—Cases in which the respiratory rates have been

shown to vary in substrates of different pH have been reported for several phyla. Hiestand and Hale (1938) found the respiratory rate of fresh-water molluscs to decrease when the pH was lowered. Hiestand (1940) found the respiratory rate of *Thyone* (Holothurian) to increase as the pH increased from 5.4 to 8.8. Von Brand (1943) found that oxygen consumption of *Eustrongylus* (nematode) remained practically unchanged in the pH range from 3.4 to 8.3. An increase in oxygen consumption was, however, observed in the two pH ranges 1.1 to 2.0 and 9.0 to 10.7. Maier and Coggeshall (1941) found that the respiratory rate of *Plasmodium knowlesi* remained constant between pH 7.0 and 8.0 but declined with further increase in pH up to 9.0.

In the present study no significant change in the rate of respiration of unsporulated oöcysts within the pH range of 1.2 to 8.8 and of sporulated oöcysts within the pH range of 4.5 to 8.8 was observed. These results are in accord with the findings of Horton-Smith and others (1940) who found that the unsporulated oöcysts of *E. tenella* could withstand exposure to sodium hydroxide (pH 11.2) for 2 hours and concluded that the effects of ammonia on *E. tenella* were due to toxic action of the drug rather than to effect of the change in hydrogen-ion concentration.

SUMMARY

1. The oxygen consumption of tissue parasitized with *E. tenella* was significantly higher than that of normal tissue.

2. The respiration of unsporulated oöcysts is from 10 to 20 times greater than that of sporulated oöcysts.

3. The respiration of oöcysts remained fairly constant within the pH range of 1.2 to 8.8.

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AMERICAN VISCERAL LEISHMANIASIS— THE ETIOLOGICAL AGENT *

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INTRODUCTION

Migone (1913) reported a case of visceral leishmaniasis from Paraguay, in an Italian who came to Brazil in 1899. In February 1911 the patient became ill with chills, fever, diarrhea and lassitude and was treated with quinine. In May he went to Asuncion, where he was found to be suffering from loss of strength and weight, anemia, leukopenia, spots on the face, back and hands, spleno-hepatomegaly, pot-belly, coated tongue, and epistaxis. *Leishmania donovani* was found in blood smears. He was treated with 0.6 gm salvarsan but died a month later with a severe attack of diarrhea. It is very likely that this patient acquired the infection in Brazil because he became sick twenty years after leaving Italy. Mazza and Arias (1926) reported autochthonous infantile kala-azar in Salta, Argentina, while Chagas and Romaña (1937) found cases in Chago, Argentina.

Penna (1934) reported that the Viscerotomy Service of the International Health Division of the Rockefeller Foundation on Yellow Fever cooperating with the National Department of Health of Brazil between March 1932 and July 1936 found 85 specimens of liver containing leishmania bodies morphologically identical with those of *Leishmania donovani*. These cases were in the states of Bahia, Sergipe, Alagôas, Pernambuco, Rio Grande do Norte, Ceará, Piauhy and Pará. Gatti, Boggino and Prieto (1939) reported an adult Paraguayan who developed kala-azar in Bolivia. Martinez and Pons (1941) described the first case of an adult with kala-azar in Venezuela. Later, Patenza and Andeze (1942) examined 842 liver specimens removed by viscerotomy and found a child infected with *Leishmania*. Cases have been found in persons between the ages of 45 days and 56 years, of whom 53.1 per cent were under 6 years, 17.4 per cent between 6 and 10 years and 29.5 per cent over 10 years of age. No evidence of epidemic incidence of the infection was found in any focus, but the disease occurs endemically and is apparently a jungle infection. The mortality was 1.8 per cent in the Amazon Valley and 0.4 per cent in the North-eastern section of Brazil.

Clinically, the evolution of the disease is very similar to that of Indian and Mediterranean kala-azar. The onset is variable, with progressive anemia, emaciation, leukopenia, monocytosis, spleno-hepatomegaly, and hemorrhagic manifestations. Death in acute cases takes place in one to three months and in chronic cases in 8 to 15 months. The parasites are easily detected in blood and by spleen and liver puncture. Cultivation on N.N.N. medium gives rise to leptomonad forms. Treatment with antimony preparations has proven specific (Chagas, 1936).

The report of the American Kala-Azar Commission (Chagas *et al,* 1938) deals with eight cases in the state of Pará in 1937, seven of which were children. Of 1,446 animals examined, seven dogs and a cat were found infected.

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Chagas *et al* (1937) claimed that by means of serological tests with immune sera, prepared by inoculating cultures into animals, the South American parasite could be differentiated from the other species of *Leishmania* and that on this account the name "*L. chagasi*" given to the parasite is appropriate. It would appear, however, that the differences noted are not greater than those which have been observed to exist among different varieties of the same species in China, India, the Sudan and the Mediterranean area. The statement that animals cannot be infected is untrue in view of the fact that da Cunha (1938, 1938a, 1938b) was able to infect the hamster, rhesus monkey and the dog, and Adler (1938) was able to infect the Syrian hamster with culture material. Another argument, namely, that the South American disease is claimed to be endemic in character in contrast to kala-azar of the Old World which is epidemic, is also invalid, since the Old World kala-azar is rarely epidemic.

Da Cunha (1938) carried out a number of serological tests with strains of *L. donovani, L. infantum, L. tropica, L. brasiliensis* and the *Leishmania* of South American kala-azar. Agglutinating sera were prepared against these organisms in rabbits. With these sera and cultures of the various strains, agglutination-absorption tests were made which indicated that "*L. chagasi*" is identical with *L. infantum* (of Mediterranean kala-azar).

Ferreira, Mangabeira, Deane and Chagas (1938) demonstrated that *Phlebotomus intermedius* became infected when fed on an infected dog, and Chagas (1939) succeeded in infecting *P. intermedius* and *P. longipalpis* by allowing them to feed on an infected dog. Later, Paranse and Chagas (1940) demonstrated that such artificially infected sandflies when emulsified and injected intraperitoneally into the hamster gave rise to generalized leishmania infection.

Adler and Theodor (1939) infected *P. papatasii* with the organism of American kala-azar by feeding the fly on suspensions of the cultured organisms. An infection rate of 24 per cent was produced in sandflies fed on culture containing 300 flagellates per 0.1 cu mm, 71 per cent in sandflies fed on emulsions of 1000–2000 per 0.1 cu mm and 89 per cent in higher concentrations. The flagellates adopted an anterior position in the sandflies.

The writer has studied the biochemical reactions of representatives of the genus *Leishmania* and of *Trypanosoma cruzi*. On the basis of the nature of their growth *L. tropica, L. donovani, L. brasiliensis* and *T. cruzi* were found to be eugonic while the dysgonic forms were *L. caninum* and *L. infantum*. All the species failed to ferment the mono- and polysaccharides, failed to produce indol, and methyl red and Voges-Proskauer tests were negative. *L. tropica* and *T. cruzi* grew in milk, failed to produce H_2S in lead acetate medium and did not liquefy gelatin. The remaining leishmanias failed to grow on these media. The thermal death point for all the leishmanias was at 40° C in 15 to 30 minutes and at 45° C immediately, while for *T. cruzi* at 40° C in $3\frac{1}{2}$ hours and at 45° C in 15 to 30 minutes. *L. brasiliensis* was insoluble in bile in 7 days, while *T. cruzi* was soluble in 96 hours, but the rest of the leishmanias were soluble in 24 hours. Colonial growth was obtained on the surface of the medium when *L. tropica* and *T. cruzi* were grown on the *Leishmania* medium (Senekjie, 1939, 1939a, 1943; Senekjie and Zebouni, 1941).

STATEMENT OF THE PRESENT PROBLEM

The object of the present study has been to ascertain the biochemical reactions,

the physical properties and the cultural characteristics of the etiological agent of American visceral leishmaniasis, and its relationship to the etiological agent of Old World kala-azar.

MATERIAL AND METHODS

The writer wishes to express his thanks to Dr. Emanuel Dias of Instituto Oswaldo Cruz of Rio de Janeiro who kindly sent cultures of *"L. chagasi."*

Leishmania-blood-agar, leishmania serum, carbohydrate medium, methyl red, Voges-Proskauer, indol, lead acetate, and milk media were prepared according to the writer's previous formulae (Senekjie, 1939, 1943; Senekjie, and Zebouni, 1941) but rabbit defibrinated blood or serum was used exclusively throughout these experiments.

The thermal death point was determined by heating saline suspensions of week-old cultures of *"L. chagasi"* in a water bath at 40° and 45° C and making direct microscopic observations for motility, and cultures at intervals of 5, 15, and 30 minutes.

The bile-solubility tests were performed by using sterile human bile obtained from the normal gall-bladder post mortem. Three-tenths cc of sterile human bile was added to 1 cc of a saline suspension of week-old cultures of *"L. chagasi."* Control cultures containing the organisms in physiologic saline solution were also prepared. The tubes were kept at room temperature, and direct microscopic observations were made at 15-, 30-, 45-, 60-minute, and at 18- and 48-hour intervals.

The egg-liver extract medium was prepared according to the formula of the writer (Senekjie, 1943).

RESULTS

Growth on carbohydrate media. The growth of *"L. chagasi"* became apparent in five days after incubation at room temperature. At first the growth was finely granular, but in 10–15 days the clumps became quite large and settled to the bottom of the tube, leaving a clear supernatant layer. On shaking the tubes, the clumps were broken up, and the suspension again became finely granular. No acid or gas was produced. The growth was eugonic. The following carbohydrates were tested in the culture media: dextrose, maltose, lactose, sucrose, mannite, dulcite, rhamnose, xylose, sorbitol, levulose, galactose, and inulin.

"L. chagasi" did not produce indol and the H_2S, methyl red and Voges-Proskauer tests were negative. In milk there was no growth.

Thermal death point. Table 1 shows the thermal death point of *"L. chagasi."* At 40° C the organism was killed in 30 minutes, but at 45° C it was killed immediately.

TABLE 1.—*Thermal death point of "L. chagasi"*

Time	40° C		45° C	
	Direct	Culture	Direct	Culture
5 min.	++	++++	−	±
15 "	−	++	−	−
30 "	−	−	−	−

Bile solubility. Table 2 shows the bile solubility of the organism. It was completely dissolved in 24 hours, while the saline controls remained motile for about six days.

TABLE 2.—*Bile solubility of "L. chagasi"*

Time	Bile	Saline
15 min.	++++	++++
30 "	++++	++++
45 "	++++	++++
60 "	++++	++++
18 hours	++	++++
24 "	−	++++
48 "	−	++++
6 days	−	+++

Colony characteristics. In 24 hours a surface film appeared. About the third day small colonies about 2 mm in diameter developed; these had an irregular contour, were slightly elevated, with a moist, shiny surface, a finely granular structure, amoeboid edges, and dirty white, transparent appearance. In about a week, the size was 3–6 mm, the colonies were irregular in shape, convex, moist, shiny and finely granular, with amoeboid "pseudopods." The colonies stopped growing about the tenth day, but progressively the surface became wrinkled, and if drying was prevented the organisms remained alive for about three weeks.

Egg-liver extract medium. *"L. chagasi"* grew well on this medium; about the fourth day the medium developed turbidity, which increased until about the tenth day. There was no surface film, but some sediment accumulated at the bottom of the slant. Usually the growth was smooth, but at times finely granular. Under the microscope longitudinal binary fission was frequently observed. Such cultures survived two weeks. By serial subcultures the organisms lived for about six generations, then stopped growing. Apparently this was due to progressive consumption of the stored growth fraction of the media. When such a scanty culture was transferred to a blood medium, the organisms grew abundantly. It was also observed

that *"Leishmania chagasi,"* and *Trypanosoma cruzi* grow well on egg-medium base with physiologic saline or Ringer's solution overlay in place of 0.5 per cent liver extract in saline.

DISCUSSION

Visceral leishmaniasis has a very wide geographical distribution. The Chinese type occurs mostly among small children but infection of adults is not rare. The dog has been proved to be a reservoir host, and the transmitting vectors are *Phlebotomus chinensis* and *P. sergenti.* In India 39½ per cent of the patients are less than 10 years old. No reservoir host has been discovered, but the vector is *P. argentipes.* The infantile kala-azar of the countries around the Mediterranean Sea mainly occurs in children below 4 years. The dog is the important reservoir host, and the vectors are *P. perniciosus, P. major,* and *P. papatasii.* The most characteristic feature of the Sudanese type is that it is resistant to treatment with antimony. The vector is *P. langeroni.* As previously stated, New World or American visceral leishmaniasis occurs in infants and adults from 45 days to 56 years of age. The reservoir host is the dog and sometimes the cat, while the vectors are *P. intermedius* and *P. longipalpis* (Ferreira *et al,* 1938; Chagas, 1939).

The writer is inclined to believe that *L. donovani,* the causative agent of the Old World visceral leishmaniasis and *"L. chagasi,"* that of American visceral leishmaniasis, are identical because of the following findings:

1. Both diseases are endemic, although Old World kala-azar may at times become epidemic (Chagas, 1936).

2. Sandflies are the intermediate hosts and transmitting agents of all types of *Leishmania. P. papatasii,* the transmitter of the Mediterranean type, is infectible with the American type (Adler and Theodor, 1939).

3. Both diseases produce generalized infection in the hamster, rhesus monkey and dog (da Cunha, 1938, 1938a, 1938b; Adler, 1938).

4. The blood picture and blood chemistry are identical in both infections (Chagas, 1936).

5. Agglutination absorption tests show that the two organisms are identical (da Cunha, 1938c).

6. The biochemical, cultural and growth requirements, as well as bile solubility and thermal death point are the same for *L. donovani* and *"L. chagasi"* (Senekjie and Zebouni, 1941).

7. The clinical course of the infection is similar in all types of kala-azar.

8. Antimony salts are specific for all types except that in the Sudan. (Kirk and Sati, 1940; Chagas, 1936).

Since *"L. chagasi,"* the etiologic agent of American visceral leishmaniasis, is epidemiologically, clinically, parasitologically, immunologically and culturally identical with *L. donovani,* the agent of Old World kala-azar, *L. chagasi* becomes a synonym of *L. donovani;* hence the former name should not be employed to designate the *Leishmania* producing American kala-azar.

SUMMARY AND CONCLUSION

1. *"L. chagasi"* is a eugonic organism which does not ferment carbohydrates, does not grow in milk, does not produce H_2S or indol, and is methyl red and Voges-Proskauer negative.

2. It is bile soluble, and is killed instantaneously at 45° C and in 30 minutes at 40° C.

3. The colony characteristics are described.

4. Evidence is provided supporting the view that *"L. chagasi"* is synonymous with *L. donovani;* hence *L. donovani* should be employed to designate the causative organism of American kala-azar.

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LYMNAEID SNAILS AS SECOND INTERMEDIATE HOSTS OF THE STRIGEID TREMATODE, *COTYLURUS FLABELLIFORMIS* (FAUST, 1917)[1]

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INTRODUCTION

From 1932 to 1941 a series of studies has been made during the summers at the University of Michigan Biological Station on the second intermediate host relations of the strigeid trematode, *Cotylurus flabelliformis*.[4] The adults of this species live in ducks. The cercariae develop in elongate daughter sporocysts in the digestive gland of the snail intermediate hosts. They escape and penetrate into snails that serve as second intermediate hosts, normally being localized in the hermaphroditic gland inside the digestive gland. In the development of the metacercaria the body of the cercaria goes through a profound metamorphosis and finally becomes an encysted tetracotyle. The developmental stages of the metacercaria of *C. flabelliformis* have never been described in detail but they appear to be like those of the closely related European species, *C. cornutus,* which have been described and figured by Szidat (1924) and Wesenberg-Lund (1934, Pl. XXV). When the cercaria penetrates into the snail it loses its tail and the body soon begins to enlarge. In about 10 to 14 days at favorable temperatures the metacercaria has grown to five or six times the length of the cercarial body, reaching about 1 mm in length, and has become very broad and thick. During this growth the internal structures appear to break down and the stellate cells of the parenchyma are widely separated by fluid-filled spaces. After maximum size is reached the tissues begin to be reorganized and condensed, the size decreases, and the hold-fast organ, the lateral cups and other structures of the tetracotyle are formed. During this reorganization the cystogenous glands become prominent, and a narrower hind-body is set off from the fore-body. Finally, the metacercaria becomes encysted and the development appears to be complete. This development takes about three weeks or more, depending on the temperature. The fully developed metacercaria (tetracotyle) is about one-third the length of the largest developing stage and is surrounded by a secreted cyst.

In order to compare the extent of development in our experiments the metacercariae counted were frequently classified into three arbitrary groups according to their stage of development, viz., (1) "developing" (all stages up to the largest and those beginning to reorganize, (2) "pre-cysts" (those with a distinct hind-body but

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[4] For a discussion of the literature on the life cycle of this species see Cort, Olivier, and Brackett, 1941.

without a cyst wall), and (3) "cysts" (those that had completed their development and were surrounded by a cyst wall).

The earlier papers of this series have dealt primarily with the influence of infections of the sporocysts and rediae of trematodes in snails on their second intermediate host relations to *C. flabelliformis*. The present paper will consider the development of the metacercariae in the lymnaeid snails of the Douglas Lake region when they harbor no infections with other larval trematodes. It is especially concerned with the presentation of the evidence that the cercariae of *C. flabelliformis* that develop in the varieties of *Lymnaea stagnalis* differ greatly in their second intermediate host relations from those that develop in the varieties of *Stagnicola emarginata*.

In our examinations of snails at the University of Michigan Biological Station we have found that three species of lymnaeids serve as intermediate hosts for the development of the cercariae of *C. flabelliformis*. Infection is widespread in the region, but usually in low incidence in *Lymnaea stagnalis appressa* Say, *L. s. perampla* Walker,[5] *Stagnicola emarginata angulata* (Sowerby), and *S. e. canadensis* (Sowerby). Single individuals of *Stagnicola palustris elodes* (Say) have also been found infected in one particular area in two separate years.

The various species of lymnaeid snails in the region serve as second intermediate hosts for the development of the metacercariae of *C. flabelliformis*. These metacercariae are widespread and abundant in natural infections in the two varieties of *L. stagnalis* and in *S. e. angulata* and *S. e. canadensis*. In one area they were also found in large numbers in *S. p. elodes*. Also, natural infections were found in at least one of the few collections we were able to make of the huge lymnaeid, *Bulimnea megasoma* (Say). All these species of snails have also been infected experimentally with the metacercariae. In addition, we obtained normal development of the metacercariae in experimental infections of *Stagnicola exilis* (Lea) and *Fossaria abrussa* (Say). The failure to find natural infections in these two species was probably due to the fact that our collections of them were from very limited areas. All these species of lymnaeid snails appear to be normal second intermediate hosts for *C. flabelliformis*, since development of the metacercariae was completed in them. They belong to four different genera of the Lymnaeidae and differ greatly in size, structure and habits. This would suggest that possibly any lymnaeid species might serve as a second intermediate host for this trematode. On the other hand, physid and planorbid snails, appear not to be normal second intermediate hosts for *C. flabelliformis* since development of the metacercariae in them only occurs when they are infected with the sporocysts or rediae of trematodes (Cort, Olivier and Brackett, 1941).

Since the species of lymnaeid snails in this region differ so much from each other it seemed of interest to compare in experimental infections the development of the metacercaria of *C. flabelliformis* in them. In setting up the experiments, examinations for natural metacercarial infection were first made of some of the snails of the collections to be used. The results of these examinations are listed under the descriptions of Tables 1 and 2 in which the results of the experiments are summarized. In carrying out the individual experiments, the snails to be infected were placed in an aquarium, and water containing cercariae of *C. flabelliformis* that had

[5] These two varieties of *L. stagnalis* are difficult to distinguish and are intermingled in the Douglas Lake region. They were used in the experiments without any attempt being made to determine to which variety each individual snail belonged.

escaped from infected intermediate hosts was poured in with them. The numbers of cercariae used and the extent of exposure varied, since frequently the cercariae were poured in over a period of several days. No attempt was made to count the cercariae that were used in infecting any particular group of snails. Therefore, the extent of exposure to cercariae could only be measured by the number of metacercariae that developed. In most of the experiments two different species of lymnaeid snails were exposed together for comparison. Also, in some of the experiments reported in this paper, snails with known infections of larval trematodes were included. The tables included in the present paper, however, give only the results for the unparasitized snails of such experiments. In every experiment some of the snails died before the date of examination. These are omitted from the tables. The experiments were terminated from about 10 days to a month or more after last exposure to the cercariae. The experiment numbers given in Tables 1 and 2 are taken from our original records and are chiefly significant in identifying experiments that are considered in more than one connection.

It was found early in the work that there are certain differences in second intermediate host relations between the cercariae of *C. flabelliformis* that develop in the varieties of *L. stagnalis* and those that develop in the varieties of *S. emarginata*. In an attempt to make these differences clear, those experiments in which the cercariae used in the experimental infections were from *L. stagnalis* are summarized in Table 1 and those in which they were from *S. emarginata* are set up separately in Table 2. The results of the experimental infections for each species of snail will be discussed separately beginning with *L. stagnalis*.

A. THE VARIETIES OF *L. stagnalis* AS SECOND INTERMEDIATE HOSTS FOR *C. flabelliformis*

The varieties of *L. stagnalis* were found naturally infected with the metacercariae of *C. flabelliformis*, often with large numbers, wherever snails of this species were present harboring the sporocysts and cercariae of this trematode. In fact, this species of snail might be considered as the type first and second intermediate host for

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EXPLANATION OF TABLE 1

A summary of the counts of metacercariae of *C. flabelliformis* in experiments in which lymnaeid snails were experimentally infected with cercariae of this trematode species that had developed in the varieties of *L. stagnalis*. All experimental snails were uninfected with larval trematodes.

Control examinations were made as follows for previously acquired natural infections with metacercariae of *C. flabelliformis* of snails from the same collections as those used in the experiments:

3A(1935) and 3B(1935) : 6 *L. stagnalis*, negative; 6 *S. exilis*, negative.
3(1937) : 15 *L. stagnalis*, negative; 15 *S. exilis*, negative.
6A(1935) : 6 *L. stagnalis*, negative; 12 *S. e. angulata*, negative.
13(1935) : 15 *L. stagnalis*, negative; *S. e. angulata*, no examinations.
5B, C, D, E (1937) : 50 *L. stagnalis*, 47 negative, 3 positive with 5 "cysts"; 23 *S. e. angulata*, 22 negative, 1 positive with 1 "cyst."
4(1937) : 20 *S. p. elodes*, 14 negative, 6 positive with 9 metacercariae.
2(1941) : 3 *B. megasoma*, negative.
4(1935) : 8 *Fossaria abrussa*, negative.
21(1938) : 25 juveniles of *S. e. canadensis*, negative.
1A and 1B(1941) : 20 juveniles of *S. e. angulata*, negative.
2(1941) : natural infections of metacercariae not found in a number of juveniles of *S. e. angulata* examined from the area from which this collection was taken.

TABLE 1

Exper. No.	Days from infection to examination	Species of snails experimentally infected	No. of snails	No. pos. for metacercariae	Data from metacercarial counts				
					Range in No.	Av. No.	Per cent "cysts"	Per cent "precysts"	Per cent "developing"
3A (1935)	25–56	*L. stagnalis*	14	14	828–3092	1742	....	...	...
	24–56	*S. exilis*	5	5	147–397	208	....	...	...
3B (1935)	35–36	*L. stagnalis*	14	14	44–444	168	97.7	1.8	0.5
	"	*S. exilis*	12	12	11–30	16	100.0	0.0	0.0
3 (1937)	17–23	*L. stagnalis*	14	14	29–289	159	88.4	10.8	0.8
	"	*S. exilis*	22	22	3–39	22	75.5	9.0	15.5
6A (1935)	7–29	*L. stagnalis*	8	8	103–1494	693	....	...	...
	7 23	*S. e. angulata*	5	4	0 21	15	....	...	...
13 (1935)	8–17	*L. stagnalis*	5	5	90–394	214	....	...	...
	"	*S. e. angulata*	21	21	1–71	25	....	...	...
5B (1937)	18–19	*L. stagnalis*	6	6	18–240	103	4.5	27.9	67.6
	"	*S. e. angulata*	10	10	3–51	22	48.4	30.9	20.7
5C (1937)	26 27	*L. stagnalis*	3	3	59–158	98	74.6	22.0	3.4
	"	*S. e. angulata*	5	5	3–55	21	21.9	41.9	36.2
5D (1937)	22–25	*L. stagnalis*	5	5	75–104	93	73.0	12.5	14.5
	"	*S. e. angulata*	8	8	1–47	23	38.7	25.7	35.6
5E (1937)	25–33	*L. stagnalis*	5	5	103- 221	158	....	...	...
	"	*S. e. angulata*	7	6	0–32	7	....	...	...
4 (1937	50–53	*S. p. elodes*	35	35	6–108	43	66.5	19.5	14.0
2 (1941)	16–18	*B. megasoma*	7	7	3- 51	16	0.0	0.0	100.0
4 (1935)	23–40	*F. abrussa*	23	23	1 87	33	....	...	...
21 (1938)	36–37	*S. e. canadensis* (juv.)	76	47	0–184	23	....	...	99 +
1A (1941)	22	*S. e. angulata* (juv.)	26	0	.....	...	.....	...	...
	"	*S. p. elodes*	4	4	14–127	49	64.9	35.1	0
1B (1941)	29	*S. e. angulata* (juv.)	25	1	6	...	....	...	...
	"	*S. p. elodes*	5	5	21–80	38	95.8	4.2	0
2 (1941)	16–18	*S. e. angulata* (juv.)	23	18	0–48	18	0	0	100.0
	"	*B. megasoma*	7	7	3–51	16	0	0	100.0

TABLE 2

Exper. No.	Days from infection to examination	Species of snails experimentally infected	No. of snails	No. pos. for metacercariae	Data from metacercarial counts				
					Range in No.	Av. No.	Per cent "cysts"	Per cent "precysts"	Per cent "developing"
2 (1936)	22–24	*L. stagnalis*	39	14	0–13	2	0.0	0.0	100.0
	"	*S. exilis*	20	20	75–208	124	36.4	18.9	44.7
10A (1935)	25	*L. stagnalis*	9	4	0–125	17	100.0	0.0	0.0
	"	*S. e. angulata*	16	16	15–414	137	0.1	1.3	98.6
10B (1935)	24	*S. exilis*	17	17	40–589	146	28.3	71.7	0.0
14 (1935)	8–18	*L. stagnalis*	5	0	.....	...	....	...	....
	"	*S. e. angulata*	15	14	0–464	199	0.0	0.0	100.0
15 (1935)	0–12	*L. stagnalis*	6	6	4–96	54	0.0	0.0	100.0
	"	*S. e. angulata*	19	19	81–785	374	0.0	0.0	100.0
5 (1936)	28–40	*L. stagnalis*	9	4	0–300	40	77.9	6.6	15.5
1 (1936)	57–59	*S. e. angulata*	6	6	279–412	302	72.9	8.6	8.5
12 (1935)	20–24	*S. e. angulata*	6	6	22–443	278	0.0	0.0	100.0
11 (1935)	24	*S. e. angulata*	2	2	44 & 62	...	0.0	0.0	100.0
	"	*F. abrussa*	30	28	0–43	12	0.0	0.3	99.7
20 (1938)	35–36	*S. e. canadensis* (juv.)	58	35	0–131	25	53.3	19.1	27.6

this trematode species, since the first description of its cercaria was from this host (Cort and Brooks, 1928) and the most complete experimental demonstration of its life cycle was made with cercariae from this host experimentally introduced into snails of this same species (Van Haitsma, 1931).

1. *Infections with Cercariae from* L. stagnalis

Our experiments (Table 1) showed that the metacercariae develop in large numbers in the varieties of *L. stagnalis* when they are exposed to cercariae of *C. flabelliformis* from this same snail species. The numbers that developed were always very much larger than those in the smaller lymnaeids in the same experiments [cf 3A and 3B(1935) ; 3(1937) ; 6A(1935) ; 13(1935) ; 5B, 5C, 5D and 5E (1937)] that had had the same exposure to the cercariae. While these differences might be interpreted as showing that *L. stagnalis* was inherently more susceptible to this infection than the smaller lymnaeids, there are obviously other factors involved. In the first place, the varieties of *L. stagnalis* used in the experiment were several times larger than the two species to which it was directly compared, *S. exilis* and *S. e. angulata.* Also, when snails of this species are irritated by the penetration of the cercariae they tend to extend their bodies out from the shell thus increasing the surface exposed to the attack of the cercariae. The results of the experiments listed in Table 1 also showed a rapid development of the metacercariae in *L. stagnalis.* In some of the snails a few "cysts" were present as early as 16 and 17 days after first exposure to the cercariae. In light and moderate infections most of the metacercariae were in the "cyst" or "pre-cyst" stages by 21 to 27 days after infection [3(1937) ; 5C and 5D(1937)]. In heavier infections development was considerably slower as shown in one experiment [3A (1935)] in which only a very small proportion of the metacercariae had become encysted by 27 days and a large proportion were still in the "developing" stages as long as 38 to 56 days after exposure to the cercariae (Table 3).

It can be seen, therefore, that the varieties of *L. stagnalis* are excellent second intermediate hosts for *C. flabelliformis* when they are infected with the cercariae that have developed in the same species of snail. Development is rapid and large numbers of metacercariae can be harbored by a single individual.

Explanation of Table 2

A summary of the counts of metacercariae of *C. flabelliformis* in experiments in which lymnaeid snails were experimentally infected with cercariae of this trematode species that had developed in *S. e. angulata* [2(1936) ; 5(1936) ; 1(1936) ; 20(1938)] and in *S. e. canadensis* [10A (1935) ; 10B(1935) ; 14(1935) ; 15(1935) ; 12(1935) ; 11(1935)]. All experimental snails were uninfected with larval trematodes.

Control examinations were made as follows for previously acquired natural infections with metacercariae of *C. flabelliformis* of snails from the collections used in the experiments:

2(1936) : 15 *L. stagnalis,* negative; 12 *S. exilis,* negative.
10A(1935) : 15 *L. stagnalis,* negative; 12 *S. e. angulata,* negative.
10B(1935) : 9 *S. exilis,* negative.
14(1935) : 6 *L. stagnalis,* negative; 16 *S. e. angulata,* negative.
15(1935) : *L. stagnalis,* no examination; 18 *S. e. angulata,* negative.
5(1936) : 2 *L. stagnalis,* negative.
1(1936) : 25 *S. e. angulata,* negative.
12(1935) : 12 *S. e. angulata,* negative.
11(1935) : 10 *Fossaria abrussa,* negative.
20(1938) : 25 juveniles of *S. e. canadensis,* negative.

2. *Infections with Cercariae from* S. emarginata

In striking contrast are the results of the experiments listed in Table 2 in which a number of snails of the varieties of *L. stagnalis* were exposed to the cercariae of *C. flabelliformis* that had developed in the varieties of *S. emarginata.*[6] Experiment 2 (1936) is a good example of the results of such infections. Of the 39 specimens of *L. stagnalis* examined 24 days after first exposure to infection only 14 contained any metacercariae at all, most of which had not even started to develop. In contrast, the 20 specimens of *S. exilis* in this experiment that had the same exposure to infection had an average of 124 metacercariae with more than half either "cysts" or "precysts." In the other experiments in Table 2 in which specimens of *L. stagnalis* were exposed to cercariae of *C. flabelliformis* that had developed in *Stagnicola emarginata* they also showed very small infections with the metacercariae [10A (1935); 14(1935); 15(1935); 5(1936)]; and, it seems probable that part of the few metacercariae that were found had come from previous naturally acquired infections. In fact, the contrast between the results of infection of *L. stagnalis* listed in Tables 1

TABLE 3.—*Records of examination of snails of the varieties of* L. stagnalis *exposed to very heavy infections with the cercariae of* C. flabelliformis *from the same host* [*Exp. 3A (1935)*]

Snail No.	No. of days from 1st exposure to examination	Total No. of metacercariae counted	No. of "cysts"	Per cent "cysts"
1	27	1633	25	1.5
2	"	828	191	23.1
3	30	1517	714	47.1
4	"	1205	506	41.9
5	33	3092	13	0.4
6	"	2168	1918	88.0
7	"	1553	650	41.7
8	38	961	633	65.8
9	"	1263	355	28.1
10	48	1570	872	55.5
11	"	2676	947	35.4
12	56	2070	979	47.3
13	"	1543	1497	97.0
14	"	2308	2308	100.0

and 2 is very striking. In the five experiments listed in Table 2, 40 of the 68 specimens of *L. stagnalis* acquired no infection with the metacercariae of *C. flabelliformis,* while in the 8 experiments listed in Table 1, all of the 60 specimens of *L. stagnalis* acquired infections, frequently of large size, that developed normally. It seems perfectly clear, therefore, that while *L. stagnalis* is a very good second intermediate host for the cercariae of *C. flabelliformis* that develop in sporocysts in this same host, it is a distinctly abnormal second intermediate host for the cercariae of this trematode species that develop in *S. e. angulata* and *S. e. canadensis.*

B. THE VARIETIES OF *Stagnicola emarginata* AS SECOND INTERMEDIATE HOSTS OF *C. flabelliformis*

In a large proportion of the areas in the Douglas Lake region from which our collections of *S. emarginata* were made, snails infected with the sporocysts and cercariae of *C. flabelliformis* were present, usually in low incidence, and a large

[6] It is interesting in this connection that in the experiments listed by Nolf and Cort in Table I of their 1933 paper, ten specimens of *L. stagnalis* in Experiment C had infections of metacercariae ranging in numbers from 402 to 1,311, although it is specifically stated that the cercariae used in infecting them came from *S. e. angulata.* It seems difficult to explain these results which are entirely different from those of all later experiments.

proportion of the snails of this species from these areas contained the metacercariae of this trematode. In collections made late in the summers they were frequently present in the juveniles as well as in the adults. Fortunately, there were a few areas where naturally acquired infections of these metacercariae were not present, from which it was possible to obtain uninfected snails for our experiments.

1. *Infections with Cercariae from* S. emarginata

Natural infections.—Natural infections with the metacercariae were sometimes extremely heavy in *S. emarginata* when exposed to the cercariae of *C. flabelliformis* from the same host. In the summer of 1933 it was found that specimens of *S. e. angulata* from a beach near Phragmites Flats on Douglas Lake had very large num-

TABLE 4.—*Records of counts of metacercariae in natural infections in unparasitized specimens of* S. e. angulata *collected from the "Phragmites Flats Area" on July 26, 1933*

Snail No.	Date of examination	Numbers of metacercariae		
		"Developing" and "pre-cysts"	"Cysts"	Total
1	8/1	688	748	1436
2	"	178	1000	1178
3	8/8	504	1057	1563
4	"	688	1033	1721
5	"	654	1360	2014
6	"	696	1263	1959
7	8/9	1130	376	1506
8	"	1112	196	1308
9	"	2011	910	2921
10	"	1172	1007	2179
11	8/14	822	633	1455
12	8/21	513	572	1085
13	"	253	1240	1493
14	"	1276	391	1667
15	8/22	862	1876	2738
16	"	50	475	525
17	"	1324	789	2113
18	"	1253	722	1975
19	"	1354	341	1695
20	"	362	455	817
21	"	504	323	827
22	"	1902	553	2455
23	"	81	204	285
24	"	332	493	825
25	"	595	462	1057
26	"	879	763	1642
27	"	8	898	906

bers of the metacercariae of *C. flabelliformis.* These metacercariae evidently came from cercariae that had developed in this same host, since the varieties of *L. stagnalis* were not found on this beach. Also, in collections of *S. e. angulata* made from this same beach during June and July, of the same year, the sporocysts and cercariae of *C. flabelliformis* had been found in 41 snails in 1,475 examined. On July 26 a collection of *S. e. angulata* was brought in from this beach. A number were immediately isolated in separate bottles for later examination. It is evident, therefore, that except for exposure to cercariae that might have escaped during the short trip across the lake from the few snails in this collection infected with *C. flabelliformis,* the metacercariae found in these snails must have been naturally acquired. Table 4 gives the counts of metacercariae from 27 snails from this collection that were not infected with larval trematodes.[7] It can be seen from the table that the counts were made over a period from August 1 to August 22. This delay in the examinations was due to the long time required to make counts involving such large numbers of

[7] The analysis of the counts of the snails of this series which were infected with larval trematodes will be given in a later paper.

metacercariae and because of the press of other work.[8] The counts are probably all less than the actual numbers because it is practically impossible to record all the metacercariae present in such heavily infected snails.

An analysis of the data of Table 4 gives an average of 1,531 metacercariae. While the range in numbers is considerable (285 to 2,921) it is certainly no greater than that in experimental infections. Only 6 of the snails had counts of less than 1,000. In the most heavily infected snails the metacercariae actually made up quite a large proportion of the bulk of the digestive gland, since they are localized in the hermaphroditic gland which lies embedded in this gland. Of the total number of metacercariae counted from the 27 snails listed in Table 4 only 49 per cent were encysted. Especially in the heaviest infections there were numerous small developing forms that were hardly larger than cercarial bodies. This suggested that the "crowding" in these heavy infections had caused a retardation of development. In fact, a considerable retardation is clearly shown from the counts of the 16 snails of this series which were kept in the laboratory in individual bottles without any chance of exposure to new infection from July 26 to August 21 and 22 before they were examined (cf Table 4, Nos. 12–27). This meant that the smallest developing forms, which were present in large numbers, especially in the snails with the largest metacercarial counts, had been in these snails for at least this period of almost four weeks without showing any appreciable development. In spite of their failure to develop these small metacercariae had not degenerated and were active at the time of examination.

Experimental infections.—The experiments in which specimens of *S. emarginata* were exposed to the cercariae of *C. flabelliformis* from this same host are listed in Table 2. It can be seen from this table that the adults of *S. emarginata angulata* in the experiments were infected with numerous metacercariae, the numbers depending on the extent of exposure to the cercariae [10A (1935); 14 (1935); 15 (1935); 1 (1936); 12 (1935); 11 (1935)]. Development appeared to be normal, although most of the experiments were terminated before development could be completed. In one experiment also [20 (1938)] juveniles were used. When examined they also contained numbers of the metacercariae that were developing normally.

From both the observations on natural infections recorded above and the results of the experiments listed in Table 2 it seems clear that *S. emarginata* both in the juvenile and adult stages is a suitable and important second intermediate host of *C. flabelliformis* when the cercariae producing the infections have developed in this same host.

2. *Infections with Cercariae from* L. stagnalis

To compare with the experiments of Table 2, we have the series in Table 1 in which specimens of *S. e. angulata* were exposed to cercariae of *C. flabelliformis* that had developed in *L. stagnalis*. There were six experiments in which adults of *S. e. emarginata* were used [6A (1935); 13 (1935); 5B, 5C, 5D and 5E (1937)]. In two of these experiments [6A (1935) and 5E (1937)] the numbers of metacercariae in the specimens of *S. e. angulata* were so small in comparison with the numbers in the specimens of *L. stagnalis* given the same exposure to cercariae as to suggest a host unsuitability. In contrast to these two, the other four experiments in Table 1

[8] The assistance of Dr. L. O. Nolf in making these counts is gratefully acknowledged.

involving adults of *S. e. anguluta* [13 (1935); 5B, 5C and 5D (1937)] infected with cercariae from *L. stagnalis* showed larger numbers of metacercariae which developed about as rapidly as those in *L. stagnalis* in the same experiments. However, the numbers of metacercariae in the adults of *S. emarginata* in these experiments were very much less than in the experiments in which adults of this species were exposed to cercariae from *S. emarginata* (Table 2). These results, therefore, suggest that adults of *S. emarginata* are somewhat abnormal second intermediate hosts for the cercariae of *C. flabelliformis* that develop in *L. stagnalis*.

In addition, there were four experiments [21 (1938); 1A and 1B (1941); 2 (1941)] in which juveniles of *S. emarginata* were exposed to infection with cercariae of *C. flabelliformis* that had developed in *L. stagnalis* (Table 1). It can be seen from this table that very few metacercariae were found in any of the juvenile snails of these experiments. In experiment 21 (1938) of the 73 juveniles of *S. e. canadensis* examined 26 were negative. Of the total number of metacercariae counted from this experiment (1645) only one was encysted and 2 were in the "pre-cyst" stage. Most of the others had not even started development during the period of 36 to 37 days from infection to examination, and a number had degenerated. In Experiments 1A and 1B(1941) only 1 of the 51 juveniles of *S. e. angulata* examined contained any living metacercariae, while the specimens of *S. p.* elodes that had had the same exposure to the cercariae contained a number of metacercariae that were developing normally. Also, in experiment 2 (1941) there was no normal development of metacercariae in the juveniles of *S. e. angulata,* since in the 23 examined all the metacercariae present were very small and most of them were about the size of cercarial bodies.

These experiments indicate that juveniles of *S. emarginata* are very abnormal hosts for the cercariae of *C. flabelliformis* that develop in *L. stagnalis*. The cercariae from this host appeared to penetrate into these juveniles in considerable numbers but most of them did not even start to develop. Juveniles of this species, even of very small size, frequently harbor natural infections of the metacercariae of *C. flabelliformis*. Also, experiment 20 (1938) (cf Table 2) shows experimentally that they are suitable hosts for the metacercariae of *C. flabelliformis* when infected with cercariae that had developed in *S. emarginata*.

C. *Stagnicola exilis* as a second intermediate host of *C. flabelliformis*

The small number of experiments in Tables 1 and 2 involving *S. exilis* indicates that this species is a normal second intermediate host for the cercariae of *C. flabelliformis* from both *L. stagnalis* and *S. emarginata*. In the three experiments in Table 1 [3A (1935); 3B (1935); 3 (1937)] this species took infections from about 10 to 14 per cent of those obtained in groups of *L. stagnalis* that were exposed to the same numbers of cercariae from *L. stagnalis*. These very light infections were probably at least partly due to the smaller size of *S. exilis* and also to the fact that these snails tend to draw into their shells when irritated by the penetration of the cercariae. In the two experiments listed in Table 2 in which specimens of *S. exilis* were exposed to cercariae of *C. flabelliformis* from *S. emarginata* [2 (1936); 10B (1935)] the infections with metacercariae were somewhat heavier than in the experiments listed in Table 1. The time of development of the metacercariae in this host was about the same as in *L. stagnalis* (Table 1) and appeared to be more rapid than

in *S. emarginata* [cf Table 2, Exp. 10A (1935) and 10B (1935)]. Some evidence of retardation of development in *S. exilis* in heavier infections is shown by Experiment 3A (1935) (Table 1) in which there was an average of 208 metacercariae.

D. *Stagnicola palustris elodes* AS A SECOND INTERMEDIATE HOST OF *C. flabelliformis*

In Table 1 three experiments included specimens of *S. p. elodes* [4 (1937); 1A and 1B (1941)]. These snails had a moderate number of metacercariae and development appeared normal. Natural infections of the metacercariae of *C. flabelliformis,* sometimes rather heavy, were found in the area from which we obtained most of the snails of this species that we collected. This area also had numerous *L. stagnalis* and specimens of this host infected with *C. flabelliformis* were frequently found. Another series of experimental infections of this species of snail are in the experiments listed in an earlier paper (Cort, Olivier and Brackett, 1941) in which it was used for infection controls. The cercariae used in these experiments were from *L. stagnalis.* In these experiments specimens of *S. p. elodes* took infections with average numbers of metacercariae from 38 to 236. Development in *S. p. elodes* seemed to be about as rapid as in *L. stagnalis* or *S. exilis.* It, therefore, is evident that *S. p. elodes* is a normal second intermediate host for the cercariae of *C. flabelliformis* that develop in *L. stagnalis.* By an unfortunate oversight no experiments were carried out in which this species of snail was exposed to infection with the cercariae of *C. flabelliformis* that developed in *S. emarginata.*

E. *Bulimnea megasoma* AS A SECOND INTERMEDIATE HOST FOR *C. flabelliformis*

Our experiments do not give much information on how satisfactory a second intermediate host this large lymnaeid is for the cercariae of *C. flabelliformis.* We have found a few naturally acquired metacercariae in this host, usually encysted, and in one experiment of Table 1 [2 (1941)] a small infection was acquired experimentally.

F. abrussa AS A SECOND INTERMEDIATE HOST FOR *C. flabelliformis*

This tiny lymnaeid took infections of metacercariae when exposed to the cercariae of *C. flabelliformis* both from *L. stagnalis* and *S. emarginata* [Table 1, 4 (1935); Table 2, 11 (1935)]. The infections were light and development though normal seemed a little slower than in some of the larger species of snails. Although *Fossaria abrussa* seems to be a normal second intermediate host for *C. flabelliformis* it is so small that any individual could hardly harbor any considerable number of metacercariae without their being greatly retarded in development due to "crowding."

DISCUSSION

The most striking point that has been brought out by this study is that there is a difference in the second intermediate host relations of those cercariae of *C. flabelliformis* that develop in *L. stagnalis* and those that develop in *S. emarginata.* The cercariae from these two species of snail intermediate hosts are alike in structure and large numbers of measurements have failed to show the slightest difference in size. Also, no differences could be found in the metacercariae and adults that developed from the cercariae from these two host species. Yet when specimens of *L. stagnalis* were exposed to the cercariae of *C. flabelliformis* from *S. emarginata* al-

most no development of metacercariae occurred. Also, the cercariae of this trematode which developed in *L. stagnalis* failed almost entirely to develop in juveniles of *S. emarginata* and developed only in comparatively small numbers in adults of this species. It appears, therefore, as if within this species two host or physiological varieties have developed which utilize different species of snails for their intermediate hosts, and which differ very strikingly in their ability to utilize certain lymnaeid species as second intermediate hosts. The finding of such differences in second intermediate host relations of cercariae of the same species from different intermediate hosts suggests a variety of problems for further experimental work. Their solution, however, will be very difficult because of the complications in the life cycle of a trematode of this type which make it very hard to carry through all phases of the cycle under laboratory conditions where experiments can be adequately controlled.

The observations and experiments described above also show that the metacercariae of *C. flabelliformis* can complete their development in all the species of lymnaeid snails that we have collected in the Douglas Lake region. Great differences in the size of infections that were acquired in snails of different species were found even when they had the same exposure to the cercariae. Since snails of the different species differed so greatly in size and in their activity when exposed to infection we could not be sure that such differences were in any case due to inherent differences in susceptibility. The single experiment in which the huge lymnaeid, *B. megasoma,* was used perhaps suggests the possibility that this snail is not very susceptible to infection. Further experiments are needed, however, to test this point. Also, there appeared to be some differencs in the time taken for the metacercariae to complete their development in the different species of hosts. Here again, the experiments are not sufficiently controlled in relation to temperature and numbers of metacercariae to draw the conclusion that there are inherent differences in this respect.

Several of the experiments showed clearly that the size of the infection has a great influence on the speed of development. In snails infected with large numbers of metacercariae there was a very marked retardation in development. In fact, in the series of *S. e. angulata* that had natural infections ranging from 1,000 to 3,000 metacercariae it appeared as if the development of some of the metacercariae was completely inhibited. It is suggested that this retardation of development in heavily infected snails may be due simply to "crowding" which produces severe competition for the available space and food supply. However, the possibility cannot be excluded that some sort of specific immunity is acquired by the host as a reaction against repeated infections with the metacercariae which may finally retard or inhibit the development of those from later infections. The close contact of the metacercariae with the tissues of the host during the whole period of their development might certainly be expected to produce some reaction. Perhaps both factors have a part in the retardation of development in heavy infections.

SUMMARY

The varieties of *Lymnaea stagnalis* and *Stagnicola emarginata* in the Douglas Lake region of Northern Michigan are common intermediate hosts for the development of the cercariae of the duck strigeid, *Cotylurus flabelliformis.* The cercariae of this species that develop in *L. stagnalis* and those that develop in *S. emarginata* appear to represent two different physiological or host varieties of this species, since they

differ in their second intermediate host relations. The variety, the cercariae of which develop in *S. emarginata,* fail almost entirely to develop into metacercariae in *L. stagnalis* while they utilize very effectively *S. emarginata* as a second intermediate host. On the other hand, the cercariae of the variety that develops in *L. stagnalis* utilize this same species very effectively as a second intermediate host, but do not develop so well in adults of *S. emarginata,* and hardly at all in juveniles. A number of other lymnaeid snails also serve as second intermediate hosts for *C. flabelliformis.* They belong to four different genera and differ greatly in size and habits. Differences in the size of the infections with the metacercariae acquired by the snails of the different species were found even when they had the same exposure to infection. These differences could be explained, at least in part, by differences in size and activity of the different snail species. It was found that in very heavy infections with metacercariae there was a marked retardation in development, perhaps due to limited space and food supply.

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STUDIES ON *DIENTAMOEBA FRAGILIS* (PROTOZOA). IV. FURTHER OBSERVATIONS, WITH AN OUTLINE OF PRESENT-DAY KNOWLEDGE OF THIS SPECIES

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I. INTRODUCTION

Since *Dientamoeba fragilis* was first described as a new genus and species of intestinal amoeba from man by Jepps and Dobell in 1918 a number of different writers have published observations and interpretations concerning it. The most comprehensive paper is that of Dobell (1940), who redescribed the organism, gave an account of nuclear and cell division, recorded observations on cultures and on transmission experiments, and pointed out similarities between *Dientamoeba* and *Histomonas*.

In several previous publications (1936, 1937, 1939, 1944) I have discussed variations in the morphology of the animal, its life history, and its possible pathogenicity. It is the purpose of the present communication to record additional observations combined with an outline of our present knowledge of this interesting amoeba.

During the past 20 years over 100 cases of infection with *D. fragilis* have been encountered in the diagnostic work of my laboratory. In each case, fixed and stained slides have been made from the feces. Most of the critical studies have been made from these slides.

For assistanc in obtaining material I am indebted to many persons, but more especially to Dr. A. D. Waltz and Dr. John H. Arnett. Dr. Sarah H. Stabler has rendered helpful technical assistance. In part, these studies have been aided by grants from the Special Research Fund of the University of Pennsylvania and from the Penrose Fund of the American Philosophical Society.

II. PROBLEMS OF DIAGNOSIS

The frequent absence of *Dientamoeba* from lists of parasites in survey reports or its mention with a very low percentage of incidence, may indicate either that the methods employed were unsuitable for its recognition or that the investigators were not sufficiently familiar with it to identify it. When the methods are applicable to cysts alone, this species will naturally not be seen. Hood (1940) noted that in a laboratory in Chicago, *D. fragilis* had not been recorded for more than 2000 fecal examinations. After a summer at the Gorgas Memorial Hospital in Panama, where experience was gained in the identification of *Dientamoeba,* an incidence of 4.2% was obtained from 164 individuals examined during the succeeding 10 months—an incidence slightly higher than that for *Entamoeba histolytica.*

For the diagnosis of *Dientamoeba,* the following are needed: 1) sufficient experience in its recognition; 2) soft or fluid feces; 3) examination while the feces are still fresh; 4) recognition of the animals in fresh smears and/or on properly fixed and stained slides. Cultures may reveal positives missed by direct examination methods.

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In feces diluted with salt solution, specimens of *D. fragilis* are spherical in shape with granular or finely alveolate protoplasm. The nuclei are not visible, but food inclusions may be. After a time rather broad, clear, thin pseudopodia may be extended, usually from only one side. In fresh smears treated with Iodine solution, the nuclei remain invisible, but the granular character of the cytoplasm persists. If fresh feces are diluted with water, these amoebae swell up and burst, releasing the endoplasm, after which the ectoplasmic shell remains as a hollow sphere (Hakansson, 1935). While this behavior appears to be characteristic I should not wish to depend upon it alone for diagnoses.

The most reliable method is to make properly fixed and stained slides. Smears of feces are commonly fixed in Schaudinn's fluid and stained with iron-haematoxylin. In such preparations, however, the nuclei often fail to stain properly. If from 15 to 20% of glacial acetic acid is added to Schaudinn's fluid, just before using, the nuclei of *Dientamoeba* generally stain much more distinctly (Johnson, 1935; Wenrich, 1937, 1941). If the usual mixture with 5% of acetic is diluted with an equal amount of distilled water, the results are often quite satisfactory. Modified Bouin's fluid (picro-formol-acetic in the proportions 75:15:10) fixes *D. fragilis* very well. It is a good routine practice to fix some smears in Schaudinn's and some in modified Bouin's. Iron-haematoxylin is the stain of choice. Haemalum stains the nuclei very well and they react positively to the Feulgen technique. Some of the many variations from the typical nuclear structure may be disconcerting, especially in the over-sized individuals with single atypical nuclei (cf. Figs. 13, 28).

It is well to prepare fixed and stained slides of all positive or doubtful cases. In our survey of 1060 students (Wenrich, Stabler and Arnett, 1935), 61.3% of the *Dientamoeba* infections were recognized on stained slides alone; and in the survey of food handlers (Wenrich and Arnett, 1942), 36.4% were detected on stained slides alone.

Culture methods may add greatly to the number of positives recognized. Many persons have recorded successful cultures of this species, for example: Cleveland and Collier (1930), Svensson (1935), Brug (1936a) and Dobell (1940). These papers should be consulted for details.

III. MORPHOLOGY AND LIFE HISTORY

1. *Size.* According to Jepps and Dobell (1918), the diameters range from 3.5 to 12 μ and most authors have repeated these figures, which are undoubtedly typical. However, both the range and the average size may vary from one population to another. In previous papers (1936, 1937) I called attention to diameters up to 17–18 μ, and Fig. 13 illustrates an individual 18 × 22 μ. These larger animals are commonly mononucleate (Fig. 13) but may be multinucleate (Fig. 10). Figs. 11 and 12 show individuals 4 μ in diameter, with 1 and 2 nuclei. Animals as small as 3 μ in diameter are sometimes seen on slides from feces. For the case described in 1937, measurements made on amoeba in 2 successive stools gave an average diameter for the first stool of 8.8 μ, somewhat less than for the second (9.36 μ). Hakansson (1936) noted variations associated with the condition of the host. When diarrhea was severe, the average diameter was greater (11 μ), than when symptoms abated. In his cultures, Dobell (1940) noted the occurrence of "giants," up to 20 μ and "dwarfs," as small as 3 μ in diameter. As noted elsewhere (Wenrich, 1944) larger

size may be correlated with polyploidy. The nucleus of the large animal shown in Fig. 9 is apparently octoploid.

2. *Cytoplasmic structure.* In living examples of *Dientamoeba,* the cytoplasm may appear to be granular or frothy. In stained specimens it often appears to be finely or more coarsely vacuolated; but there are many variations from the typical alveolar condition. Larger vacuoles may be few (Fig. 4) or many (Fig. 20). Occasionally there is one large central vacuole (Fig. 29). Extensive vacuolation represents a degenerate condition. Homogeneity is another. This may affect patches here and there (Figs. 9, 10, 28), the endoplasm alone (Fig. 26), or the entire cytoplasm (Figs. 11, 17, 25). Figs. 23 and 24 apparently represent intermediate conditions.

In stained specimens there may or may not be evident a line of demarcation between ectosarc and endosarc. The degree to which this character finds expression varies with individuals, with populations, and doubtless varies with the conditions of the environment at the time the animals were fixed. In some cases the ectosarc is very sharply demarcated (Figs. 13, 26, 27, 30, 39). The relative width varies greatly; in Figs. 26 and 39 it is narrow, wider in Figs. 27 and 30. In these last two figures the zone of contact between the ectosarc and endosarc is deeply stained. In Fig. 31 a similar narrow, deeply-stained zone surrounds a small area of cytoplasm containing one nucleus. In another example (not illustrated) each nucleus was similarly enclosed in an island of cytoplasm marked off by a deeply stained boundary. In Fig. 30 the ectosarc has a peculiar radial organization. The deeply staining boundary layer probably plays an important role in the activities of the animal.

The food inclusions seen on prepared slides are diverse, as indicated by the few examples illustrated in Figs. 32–40. Various types of bacteria are found, including chains (Fig. 32) and filaments (Fig. 36); also yeasts (Fig. 33), *Blastocystis* (Figs. 35, 40), starch grains (Fig. 34), and unidentifiable parts of cells (Figs. 37, 38). Cannibalism sometimes occurs (Fig. 39), and the parasite, *Sphaerita,* is not uncommon (Fig. 40). There seems to be less hypertrophy in cells of *Dientamoeba* parasitized with *Sphaerita* than in some other endozoic amoebae. Dobell (1940) noted the avidity of these organisms for starch grains in culture and also that they ingested red blood corpuscles.

The methods of ingestion appear to be similar to those described for *Entamoeba muris* and *E. ranarum* (Wenrich, 1941a). Clusters of bacteria may be taken in by broad food cups (Fig. 41), while a rod or a filament appears to be ingested through a narrow opening which may (Fig. 43) or may not (Figs. 42, 44) be accompanied by a deeply stained "pharynx."

In my study of *Histomonas* from wild ring-neck pheasants (1943), tube-like extensions of the cytoplasm were found in about 15% of the animals on stained slides. Fig. 49 illustrates one of these. It will be seen that the walls of this extension are made up of the layer of cytoplasm that lies between the ectosarc and the endosarc. Somewhat comparable structures have been found in *Dientamoeba* (Figs. 45–48). They may be broad (Figs. 46, 47), apparently containing food elements, or may be relatively narrow (Figs. 45, 48). These differentiations may have extensive internal components, as shown in Figs. 45 and 46. They are much more common in some populations than in others.

3. *Nuclear number. Dientamoeba* is characterized by the presence of 2 nuclei

in a great majority of the individuals in most cases; but the percentage varies greatly in different populations. Jepps and Dobell (1918) recorded an average of about 80% of binucleates and this is apparently typical. But Kudo (1926) reported only 12.2% of binucleate amoebae in the case that he studied. Different stools from the same host give different percentages. In the case of the child suffering from ill health described in the second of these studies (1937), the binucleates in 5 different stools were only 73, 38, 9, 46 and 57%. Since many of the mononucleates, when they are in the majority, are larger than usual, there may be a correlation between monoclearity and symptoms, as well as between size and symptoms (cf. Hakansson, 1936).

Various authors have reported the occasional presence of more than 2 nuclei in an individual (cf. Dobell, 1940). Many populations in my collection of slides seem to lack such supernucleates, while in others they are not uncommon. Figure 10 represents an individual with 7 nuclei, one larger than the others; on the same slide, one other with 7, one with 5 and about a dozen with 3 or 4 nuclei were observed.

4. *Nuclear size.* Nuclear size tends to be correlated with cell size but there are many exceptions in some populations. Most nuclei range from 0.8 to 3 μ in diameter, but in oversized animals, they may be larger. In figure 13 the nucleus is 4 μ and in figure 20 it is 5 μ in diameter. These are, of course, abnormal specimens.

5. *Nuclear structure.* According to the original describers, the structure of the nuclei is vesicular, there being a central chromatic area called the "karyosome," composed of a number of small chromatin granules embedded in a weakly staining matrix; surrounding the "karyosome" there is a clear area enclosing which is the delicate nuclear membrane with little or no chromatin on its inner surface. A few fine fibers radiate out from the "karyosome" to the nuclear membrane and small granules may be seen at the point of contact with the membrane.

Most other authors have agreed to this description but some have noted considerable deviations from it (cf. Wenyon, 1926, and my previous papers). On the basis of material from cultures, Dobell (1940) has revised the description, stating, among other things, that neither matrix nor radiating fibers are seen. Kudo (1926) and others have verified the existence of the radiating strands and elsewhere (1944) I have interpreted them as chromosomal fibers, comparable to those reported by Cleveland et al (1934) for certain hypermastigote flagellates. Apparently culture amoebae do not show some details of structure as clearly as do those from feces.

In my series of papers on this species I have called attention to the great amount of variation in the appearance of the nuclei as observed on fixed and stained slides. To the variations previously described, Dobell (1940) has added a "true 'resting' condition" in which there is a prominent endosome with the remainder of the chromatin dispersed through the nuclear space and up against the nuclear membrane (cf. Fig. 4). The formation of such a "resting" condition would, theoretically, be a normal process, but I have found this stage mostly in populations showing abundant evidence of degeneration, and failed to find it in an apparently normal population in which many individuals were undergoing nuclear division. Therefore, it seems doubtful that it is a necessary part of the nuclear division cycle.

Post-divisional changes in the nucleus appear to be of two general sorts: A) those which take place within the central area (= the original "karyosome" of Jepps and Dobell); and B) those during which the chromatin spreads out through the

nuclear space to the membrane to produce the "resting" condition. Under the first category (A) may be noted several types of changes: 1) the characteristic condition is for one or more of the 4 telophase chromosomes to divide once to produce chromomeres which become somewhat diffuse in appearance. One of these chromosomes may fail to divide, retaining its staining capacity to become the endosome (Figs. 14, 39). This latter may also divide, in which case the endosomes may be very difficult to identify (Figs. 24, 31); 2) successive divisions may give rise to more than 8 chromomeres or "granules" (Figs. 1, 17; diploidy is an alternative interpretation here); 3) 3 of the 4 telophase chromosomes may become more diffuse without dividing while the fourth remains as a more deeply stained endosome (Figs. 23, 26, 27, 38, etc.); 4) the "granules" may lose their identity in a diffuse mass (Figs. 15, 16); 5) the chromatin may become resolved into a reticulum (Figs. 2, 13).

Dispersion out to the nuclear membrane (category B, above) may apparently take place in different ways. As previously shown (1944) dispersal may be radial from the central cluster of granules to the nuclear membrane; succeeding this, or possibly as an alternative method, a reticulum is formed with an endosome remaining (Fig. 3). In either case, eventual disperson may be fairly complete and either uniform (Fig. 36) or with a well-defined layer against the membrane (Fig. 4). It is possible that some of the conditions listed under A, above, might become transformed into this final stage of the "resting" condition, but the evidence indicates a difference between categories A and B.

When dispersion takes place according to one or the other of the first 4 types in category A, the endosome appears to be derived from one of the 4 telophase chromosomes. This would be in harmony with the now generally accepted idea that at least one type of nucleolus in metazoan cells is derived from special loci in particular chromosomes. Chen (1936) showed definite relationships between nucleoli and certain chromosomes in another protozoön, *Zelleriella intermedia.* In the types of dispersion mentioned in category B, the evidence points to the origin of the endosome from more than one, possibly all the chromosomes, as seems to be the case for the "chromocenter" in certain kinds of metazoan nuclei. Apparently the 2 types of endosomes (nucleoli) do not appear in the same nucleus in *Dientamoeba,* but our information on this point is incomplete.

Dobell (1940) mentions individuals with 2 endosomes in the nucleus. In one population I found a considerable number of these (Fig. 5) and even one with 4 endosomes. I have interpreted the additional endosomes as evidence of diploidy and tetraploidy. Fig. 9 shows an oversized individual containing a hypertrophied nucleus with 8 endosomes, presumably a case of octoploidy.

Some of the more extreme types of dispersion mentioned above may represent degeneration. In one population nearly all the nuclei, in both binucleates and mononucleates were in a condition similar to that in Fig. 15, probably all degenerate. Another kind of nuclear degeneration appears in the form of pycnosis. Figs. 6, 7 and 8 are from a slide on which most of the members of the population are normal, but some show the pycnotic nuclear condition illustrated in Figs. 7 and 8. Fig. 6 presents an intermediate condition. In Fig. 25 is depicted an apparently degenerating polyploid nucleus in a state of arrested division. It is, of course, impractical to illustrate and describe all the different degenerative conditions.

The kind of animals that survive longest in feces should be a matter of interest.

Figs. 14–20 illustrate individuals from feces kept at room temperature for 24 hours, and Figs. 21 and 22, specimens from 48-hour-old feces. One could argue that they must be fairly normal to be able to live that long, or, conversely, that they must be abnormal after having lived that long in a supposedly unfavorable environment. For the most party they appear to be abnormal, but the 48-hour survivor represented by Fig. 22 appears to be fairly normal. The nuclei in Figs. 15 and 16 are doubtless abnormal. In Fig. 18 one nucleus is fairly normal while the other is hypertrophied and abnormal. The nucleus in Fig. 19 shows 4 prophase chromosomes, probably not normal. That in Fig. 20 contains 8 "granules," 6 of which are seen to be double; the other 2 may also be double but not oriented so as to reveal their true condition, or they might be endosomes. This is probably a diploid animal with a hypertrophied nucleus and cell body.

6. *Nuclear division.* For *Dientamoeba,* nuclear division has been incompletely described by Kudo (1926), Brug (1936), Dobell (1940) and Wenrich (1939, 1944). Relatively few details are provided by Kudo and Brug and the fuller accounts by Dobell and Wenrich are admittedly incomplete. Dobell's description is based upon culture material while mine are based on slides from feces. Differences in our interpretations may be due, in considerable measure, to this difference in source.

Nuclear division is mitotic. Dobell (1940) believes that the chromosome number is 6, but I have shown that it is 4 (1939, 1944). There is one smallest, one largest, and 2 of intermediate size. They show a longitudinal split at mid-prophase. Dobell states that the desmose is extranuclear. I first described it as intranuclear (1939) but later discovered a centriole on the nuclear membrane surrounded by a small astral area. This divides to spin out the desmose which comes to lie near the center of the spindle in late prophases, metaphases and early anaphases. It may possibly fit into a fold in the nuclear membrane, as in certain flagellates (cf. Cleveland et al, 1934). In late anaphases it takes a more peripheral position and, after nuclear constriction, persists as a strand connecting the daughter nuclei for a relatively long time, usually until plasmotomy occurs. Unbalanced anaphases, seen on some slides, offer a clue to the inequality of nuclear size observed in occasional individuals.

7. *Cytoplasmic divisions.* Dobell (1940) described cytoplasmic division in living animals from his cultures. The cell elongates and constricts into 2 approximately equal parts, each containing one nucleus. Both Dobell and I find that animals undergoing division usually display a persisting desmose, as revealed by stained slides. I have records of only 2 individuals without desmoses which were elongated and constricted, so that apparently such animals seldom divide. At present there is no evidence that polynuclear animals with 3, 4 or more nuclei are capable of cytoplasmic division.

8. *Viability in feces.* Aside from its multiplication by binary fission the life history of *D. fragilis* is very incompletely known. Jepps and Dobell (1918) in their original description noted its rapid disappearance from feces but stated that degenerate individuals with large central vacuoles (cf. Fig. 29) might live in that condition for 2 or 3 days at room temperature. Temperature does affect survival as is indicated by the following observations. In Feb., 1927, a stool containing large numbers of *Dientamoeba* was obtained within 3 minutes after deposition. A portion was placed in a covered dish on the ledge outside the window where the temperature

was 3.5° C. At the end of 2 hours practically all the animals had disappeared, while in the portion kept at room temperature there was no noticeable decline in numbers, and 23 hours later there were still some to be found. In the first of these studies (1936) I called attention to the survival of considerable numbers in feces kept for 24 hours at summer room temperature (August, 1935) (cf. Figs. 14–20), and some few in 48-hour-old feces (Figs. 21, 22). Hakansson (1936) noted survival at room temperature for 48 hours in stools with much starchy residue, and for 3 or 4 days in saline smears sealed to the slides with vaseline. It is obvious that *D. fragilis* does not always disappear from stools as quickly as earlier reported.

Mononucleate animals seem to survive longer in feces than binucleates. On slides made at the time of the original examination in the case mentioned above (August, 1935), 22% of the animals were binucleate. On slides made 24 hours later only 9% showed 2 nuclei. This would suggest that binucleate animals were disappearing faster than the mononucleates, though division of some of the binucleates might have occurred. On one of the earlier slides, measurements of 100 individuals gave a range in diameter from 5 to 12 μ with an average of 7.9 μ. Only one was as much as 12 μ in diameter. On a slide from the 24-hour-old feces measurement of 100 animals gave a range of diameters from 5 to 15 μ with an average of 9.07 μ. Eight specimens were 12 μ or more in diameter (cf. Figs. 14–20). The average diameter of the binucleates was 10 μ indicating that they had shared in the size increase. Similar results were obtained from another stool from the same host when kept at room temperature for 24 hours. These observations suggest that there was an actual increase in size during the 24-hour period.

9. *Absence of cysts.* Nearly all students of *Dientamoeba* have recorded failure to find cysts. Kofoid (1923) reported cysts but an examination of his figures indicates that he was observing animals with thin clear ectosarcs (cf. Fig. 26). In the first of these studies (1936) I suggested that small deeply-stained individuals (cf. Figs. 11, 17, 23–25) might be "pseudocysts," having in mind the rounded-up and cyst-like stages of some species of *Trichomonas*. It seems more likely, however, that these represent one form of degeneration. They appear to be no more common in 24-hour old feces (Figs. 14–20) than in fresher material.

IV. RELATIONS TO HOST

1. *Incidence.* As noted by Dobell (1940) and others the geographical distribution of *D. fragilis* is world-wide. Its small size, absence of cysts, rapid disappearance from feces and difficulty of recognition readily account for the delay in its description until 1918 (Jepps and Dobell). It is somewhat surprising and disappointing that, since the publication of the original description, reports of so many surveys fail to record its presence or report only a very small percentage of infection. A review of 19 surveys made in the United States, during the past decade, in Washington, D. C., Georgia, Florida, Louisiana, Mississippi, Kentucky, Indiana, Missouri and California, indicates that 65,253 persons were examined without recording the presence of *Dientamoeba* in any of them. Seven other surveys, made in the same decade, in So. Carolina, Kentucky and California, record the examination of 19,001 persons with an average incidence of 0.64%, the highest recorded being 3% in 678 children in a Los Angeles hospital (Kessel and Sinitsin, 1938).

In Philadelphia, Pa., Wenrich, Stabler and Arnett (1935) found an incidence

of 4.3% in 1060 students, when only one normally passed stool per person was examined. Employing the same methods, Wenrich and Arnett (1942) found 3.9% in 190 food handlers, but as a result of more than one examination for some of these individuals, an incidence of 7.4% was obtained. Stabler (1941) reported 2.8% in 106 students taking his parasitology course (one examination per person). Hinshaw and Showers (1934) found 0.3% in 368 patients at the University of Pennsylvania hospital; while Rothman and Epstein (1941) reported cases in their private practice they gave no percentage, but Rothman and Laskey (1943) found 2.3% in 306 employes of a large hospital. There is no reason to suppose that *Dientamoeba* is more prevalent in Philadelphia than in other similar communities.

In certain other studies, incidences recorded have been relatively high. Svensson (1928) found 8.7% in formed stools from 46 asylum patients, but the incidence was increased to 23.9% when purged stools were examined. Andrews (1934) in his study of the relative advantages of normal vs. purged stools found 3 cases in 50 persons (6%). Cleveland and Collier (1930) discovered 6 infections in 100 persons when culture methods were employed. Brug (1936a) reported 12% incidence in 80 asylum patients by direct examination methods but increased this to 36% by the use of cultures. Hood (1943) obtained incidences of 4.8% and 7.5% for 1126 hospital patients and 265 hospital employes, respectively, examined at a Chicago hospital from 1938 to 1942 during which years iron-haematoxylin smears were made in addition to other routine methods. For *Entamoeba histolytica* the incidences were only 3.46% and 1.88% for the same groups. When properly trained observers employ appropriate techniques, the incidence of *Dientamoeba* will be found to be much higher than present survey results indicate.

A high incidence of infection with *D. fragilis* has been reported for the inmates of certain institutions for mental patients. For example, more than 50% in an asylum in Sweden (Svensson and Linders, 1934); 36% in an asylum in Holland (Brug, 1936a); and 42% in an asylum in Panama (Hakansson, 1937). On the other hand, Young and Ham (1941) reported only 1% in 142 mental patients in South Carolina and Burrows (1943) only 0.1% in 1418 patients at the same institution. Kmecza (1939) failed to find any *Dientamoeba* in 1200 patients in a hospital in Indiana as did Hopp (1944) in 771 patients in another hospital in the same state. The experience of Hood (1940, 1943) in Chicago, would suggest that *Dientamoeba* is to be found in that section of the country if care is taken to identify it.

2. *Possible pathogenicity.* Evidence that *Dientamoeba* may sometimes be a pathogenic agent has been presented from time to time, for example by Gittings and Waltz (1927), Hakansson, (1936), Wenrich (1937), Sapero (1939) and Hood (1940). It may possibly be significant that of the 8 original cases listed by Jepps and Dobell (1918), 6 were soldiers with a history of dysentery or other disturbance of the digestive tract. Another was a native of Britain who had never been abroad, but *Dientamoeba* was found when he was suffering from an attack of diarrhea attributed to a chill. The eighth case was the one in whose feces Wenyon had observed this amoeba. Gittings and Waltz reported 2 cases of children with poor health and a high percentage of eosinophiles accompanied by infection with *D. fragilis*. Hakansson reported an infection in an adult in Panama where the presence of large numbers of *Dientamoeba* was associated with aggravated diarrhea and other gastro-intestinal symptoms. Both the symptoms and *Dientamoeba* disappeared with carbarsone treatment.

Wenrich (1937) gave a more complete history of "case 1," of Gittings and Waltz, which later developed liver abscess. Unfortunately no microscopic examination was made of the exudate from the abscess when it was drained, and the patient moved away making further study impossible. With Dr. Waltz a number of other children with poor health and infection with *D. fragilis* were studied. Some, but not all, also had high eosinophilia.

Sapero (1939), in a report on non-dysenteric amoebiasis among 450 U. S. Navy men, recorded that of 106 who harbored *Entamoeba histolytica,* 43.4% had complaints; of 108 who had no intestinal Protozoa, only 7.4% indicated symptoms; and of 236 who had other intestinal Protozoa, 16.1% had complaints. Hosts of *Entamoeba coli, Endolimax nana* and *Iodamoeba bütschlii* showed no more symptoms than those without Protozoa. On the other hand, complaints were recorded for 27.3% of 44 cases of infection with *Dientamoeba,* "A finding which suggests a pathogenic rôle for this organism."

Hood (1940) found 7 cases of *D. fragilis* infection, 3 children and 4 adults, among 164 persons examined. One of the children, in whom *E. histolytica* was also found, had definite intestinal disturbances. Another child had diarrhea and 11% of eosinophiles. The other child's mother reported that it had had worms. The 4 adults had diarrhea and other intestinal symptoms. One patient left the hospital before treatment was instituted, but for the other 3, treatment removed both the amoebae and the symptoms. Wenrich, Stabler and Arnett (1935) found that the students who harbored *Dientamoeba* reported somewhat more gastro-intestinal disturbances than those who were infected with *E. histolytica.*

I have had 4 periods of infection with *D. fragilis* lasting 5 months, 2 years, 2 years and 2 months, and 4 months, respectively. There was a 2-year interval between the first and second periods and between the second and third, and a 7-year interval between the third and fourth periods. *Dientamoeba* has not appeared during the past 7 years. For the first period, *D. fragilis* was associated with *Endolimax nana* throughout and with *Chilomastix* for a short time. During the second and third periods no other protozoön was found. For the fourth period, *E. nana* and *Enteromonas* were also present and still persist. At the beginning of each period, *Dientamoeba* was discovered when a stool was examined because of an attack of diarrhea. In each infection the diarrhea gradually abated but there followed a period of increased fatigability and low resistance to colds. No treatment was ever taken for the infections and the organisms disappeared spontaneously.

There is, of course, no definite proof of pathogenicity in all this "evidence," but the suggestion that this amoeba can play a pathogenic rôle in some persons is very strong. Since most of the hosts of *Entamoeba histolytica,* at least in temperate climates, are carriers showing no very obvious symptoms, it is probable that a still larger percentage of hosts of *Dientamoeba* are also carriers without symptoms. At present there is no evidence that it invades tissues but it appears to be associated with more generalized evidences of ill health suggesting that the effects are toxic in nature. More studies will be needed to determine whether or not *D. fragilis* can be the cause of illness, but an effort to settle the question should be made.

3. *Transmission.* The mode of transmission of *D. fragilis* from host to host is still unknown. There is evidence that, in more temperate climates, at least, it has an incidence as high as, or higher than, that for *Entamoeba histolytica.* It shares

with other parasites an unusually high incidence among patients in mental asylums. The familial factor may be operative since Hakansson (1937) reported infections in 4 members of a Panamanian family of 9, and in all members of a white family of 4. One might therefore assume that this amoeba would be transmitted by contamination of food and drink as are other intestinal Protozoa; but of this we have little knowledge. Dobell (1940) swallowed a culture of *Dientamoeba* but remained negative. Equally negative were the results of his attempts to infect two monkeys by feeding them cultures and by injecting one per anum. Perhaps these culture amoebae were low in invasive capacity. Dobell also tried to infect 9 young chicks per anum and obtained a temporary (7-day) infection in only one of them.

Infections in other animals have been reported. Hegner and Chu (1930) found *Dientamoeba* in 2 out of 44 wild monkeys (*Macacus philippinensis*) examined in the Philippines, and Knowles and Das Gupta (1936) recorded an infection in another monkey (*Silenus irus*) examined in India. Regandanz (1936) reported its persistence for 15 days in one of 4 rats injected rectally with human feces containing *D. fragilis*. I fed *Dientamoeba* in milk to a rat but got no infection.

Hakansson's (1935) observations that these amoebae swell and burst when placed in water indicates that they are not transmitted by contaminated water. At various times I have placed feces containing *D. fragilis* in boiled pond water or in boiled hay infusion, but got neither survival nor cysts. The absence of cysts makes the problem of transmission a very difficult one.

Dobell (1940) believes that *Dientamoeba* is closely related to *Histomonas,* which may be transmitted by the eggs of the caecal worm, *Heterakis gallinae.* He suggests that *D. fragilis* might be similarly transmitted, possibly by *Trichuris trichiura* or by *Ascaris lumbricoides.* Experiments are needed to test this hypothesis.

V. COMPARISON BETWEEN *Dientamoeba* AND *Histomonas*

Dobell (1940) suggests that *D. fragilis* has many of the characteristics of a flagellate. *"Dientamoeba* is, indeed, a typical flagellate except for the important circumstance that it possesses no flagella." He cites similarities to *Histomonas meleagridis,* as follows: *Histomonas* grew in all the media that he used for *Dientamoeba;* it ingests red blood corpuscles in cultures, as does the latter; some of the nuclei contain a group of chromatin granules reminiscent of those of *Dientamoeba,* while others have a "resting" stage like that of the latter species; during division the desmose is a conspicuous feature and may persist until cell division in both species; Tyzzer's (1934) figures (Plate IV) of living *Histomonas,* except for the flagella on some individuals, could pass as those of *D. fragilis.*

To these similarities may be added from the present study, the formation of the peculiar tube-like cytoplasmic differentiations on both organisms, and associated with this the (often) deeply stainable boundary zone between the ectosarc and endosarc. Another flagellate character is the presence of chromosomal fibers in interphase and prophase nuclei.

However, since *Dientamoeba* has never been known to develop flagella, it must still be classed as an amoeba. On the other hand, it is sufficiently distinct from other amoebae to be placed in a family by itself; but I doubt if any useful purpose would be served by introducing a new family name into the literature at this time.

VI. SUMMARY

1. Over 100 cases of infection with *D. fragilis* have appeared in diagnostic work of my laboratory. Fixed and stained slides have been made in each case. Most of the critical observations have been made on these slides.

2. For diagnosis, training in recognition is required. Fresh soft feces should be examined. Cultures are useful in detecting positives. Properly fixed and stained slides should be made for all apparently positive or doubtful cases. From 15 to 20 percent of glacial acetic acid added to Schaudinn's fluid increases the stainability of the nuclei. Picro-formol-acetic in the proportions of 75:15:10, fixes this species very well. Iron-haematoxylin is best for staining.

3. Diameters range from 3 to 20 μ; most being from 5 to 10 μ. Larger individuals are usually mononucleate but may be multinucleate. Nuclear structure in larger mononucleates is commonly atypical making identification difficult.

4. The cytoplasm is typically finely alveolate or granular in appearance. The ectoplasm may be demarcated from the endoplasm by a deeply stainable zone, which is probably related to important activities of the cell. In degenerate animals one to many large vacuoles may be seen. Degenerate cytoplasm may become homogeneous in patches, in the endoplasm alone, or in the entire cell.

5. Food bodies include bacteria of many kinds, yeasts, *Blastocystis,* cellular fragments and starch grains. Ingestion of red blood cells has been reported. Cannibalism may occur and parasitization by *Sphaerita* is common.

6. Tube-like extensions of the cytoplasm, comparable to those reported for *Histomonas,* occur also in *Dientamoeba.*

7. Although most populations have about 80 per cent of binucleate individuals, there is much variation; in one population only 9 per cent had two nuclei. Supernucleate animals with 3 and 4 nuclei occurred occasionally in some populations; others with 5 to 7 nuclei appeared less frequently.

8. Nuclei of normal individuals vary from 0.8 to 3 μ in diameter. Large mononucleates may have nuclei 4 or 5 μ in diameter. Variations in nuclear structure are notable. Typically there is a central area containing 4 to 8 "granules" or chromatic lumps. One is usually more heavily stained, being designated the endosome. Dispersive processes are varied and may lead to a "resting" condition with a distinct endosome and finely divided chromatin out to and against the nuclear membrane. Among nuclei of this type two endosomes are not uncommon in some populations; 4 and 8 endosomes have been seen. These are interpreted as representing diploid, tetraploid and octoploid conditions. Extreme forms of disperson may be degenerate. Nuclear pycnosis is another degenerative condition.

9. During nuclear division 4 chromosomes divide on a spindle which includes a desmose stretched between polar centrioles. In the early prophases the centriole has a small astral area around it. In midprophase the chromosomes show a longitudinal split. After nuclear constriction the desmose persists for a relatively long time, commonly until cytoplasmic division takes place.

10. Although these amoebae disappear rapidly in feces, some may survive 48 hours or more at summer room temperature. At 3.5° C they disappeared in 2 hours. Survivors are more commonly uninucleate and larger than usual.

11. Most authors are agreed on the absence of cysts for this species.

12. When properly trained observers employ appropriate methods, the incidence of *Dientamoeba* as revealed in surveys may be as great as, or greater than, that of *Entamoeba histolytica*. Several observers have reported unusually high incidences in the inmates of mental asylums.

13. Evidence is accumulating that *Dientamoeba* can be a disease producing agent in some instances. More studies are needed to determine clearly its possible pathogenic potentialities.

14. The mode of transmission is as yet unknown. Dobell (1940) was unable to infect himself or two monkeys with culture amoebae. He suggests that it may be transmitted by the eggs of some nematode worm, as is true of *Histomonas meleagridis*.

15. Dobell (1940) called attention to the similarities between *D. fragilis* and *Histomonas meleagridis*, such as general appearance, nuclear structure and the persistence of the desmose. Additional similarities recorded here are: 1) the deeply-staining zone between the ectosarc and endosarc, and 2) the formation of tube-like extensions from and within this zone. Another flagellate character is the presence of chromosomal fibers in the interphase and prophase nuclei. However, since *Dientamoeba* has never been observed to produce flagella, it must still be classed as an amoeba. On the other hand, it is so different from other amoebae that it might well be placed in a separate family.

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VIII. EXPLANATION OF PLATES AND FIGURES

All figures have been drawn with the aid of a camera lucida at an initial magnification of X3000 and have been reduced about 1/3 in printing. All are from prepared slides which had been fixed in Schaudinn's fluid, with 5 to 20 per cent of acetic acid added, or with picro-formal-acetic in the proportions of 75:15:10. All were stained with iron-alum haematoxylin.

PLATE I, p. 336

FIG. 1. Individual with about 16 "granules," besides the endosome, in the central nuclear area.
FIG. 2. A reticulum has formed in the central nuclear area.
FIG. 3. The chromatin radiates out from the endosome and forms a reticulum which reaches to the nuclear membrane.
FIG. 4. Nucleus in the "resting" condition.
FIG. 5. Nucleus has dispersed chromatin and 2 endosomes.
FIG. 6. Early stage in nuclear pycnosis.
FIGS. 7 and 8. Mononucleate and binucleate examples with pycnotic nuclei.
FIG. 9. Oversized individual with a large nucleus containing 8 endosomes.
FIG. 10. Large individual with 7 nuclei, one larger than the others.
FIG. 11. Small mononucleate with homogeneous cytoplasm, 4 μ in diam.
FIG. 12. Normal-looking binucleate, 4 μ in diam.
FIG. 13. "Giant," 22×18 μ, with nucleus 4 μ in diam.
FIGS. 14–20. Specimens from feces kept 24 hrs. at summer room temperature.
FIG. 14. Nucleus has 6 "granules" besides the double endosome.
FIG. 15. Chromatin diffuse in the central nuclear area; 1 endosome.
FIG. 16. Chromatin diffuse in central area; 2 endosomes.
FIG. 17. Animal with homogeneous cytoplasm and 2 nuclei, each with more than 8 "granules."
FIG. 18. Animal with 2 nuclei different in size.
FIG. 19. Nucleus contains 4 prophase chromosomes.
FIG. 20. Nucleus, 5 μ in diam., contains 8 "granules"; probably diploid.
FIGS. 21 and 22. From feces kept at summer room temperature for 48 hrs.
FIG. 21. Animal with degenerate-looking nucleus and cytoplasm.
FIG. 22. Animal with fairly normal nuclei and cytoplasm.

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PLATE II, p. 338

FIG. 23. Nuclei look normal; cytoplasm approaching homogeneity.
FIG. 24. Nuclei look degenerate; cytoplasm approaching homogeneity.
FIG. 25. Cytoplasm homogeneous; nucleus, apparently polyploid, in a state of arrested division.
FIG. 26. Endoplasm homogeneous; ectoplasm distinct; nuclei look normal.
FIG. 27. Sharply defined boundary between ectosarc and endosarc, each of which has distinctive structure.
FIG. 28. Large mononucleate with large patch of degenerate cytoplasm.
FIG. 29. Cytoplasm has large central vacuole.
FIG. 30. Ectosarc thick, with radial organization.
FIG. 31. Deeply staining "boundary layer" surrounds small area of cytoplasm containing one nucleus.
FIGS. 32–39. Animals with various types of food inclusions.
FIG. 32. A bacterial chain besides other food bodies.
FIG. 33. Contains a yeast cell.
FIG. 34. Contains 2 starch grains.
FIG. 35. Contains 3 specimens of *Blastocystis.*
FIG. 36. Animal has ingested a long filament.
FIGS. 37 and 38. Contain unidentificable parts of cells.
FIG. 39. Cannibalism; a large one has ingested a smaller one.
FIG. 40. Contains 2 specimens of the parasites, *Sphaerita;* 1 of *Blastocystis.*
FIG. 41. Large food cup partially enclosing cluster of bacteria.
FIG. 42. Long rod, mostly ingested.
FIG. 43. Rod being ingested; note deeply-stained "pharynx."
FIG. 44. Ingested rod or filament.
FIG. 45. "Cytoplasmic differentiation"; cf. fig. 49.
FIG. 46. Combination of "cytoplasmic differentiation" and food cup.
FIG. 47. Partly extended tube-like "differentiation."
FIG. 48. Projecting narrow "differentiation."
FIG. 49. Example of *Histomonas* from ring-neck pheasant, showing tube-like "differentiation" from layer between ectosarc and endosarc.

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